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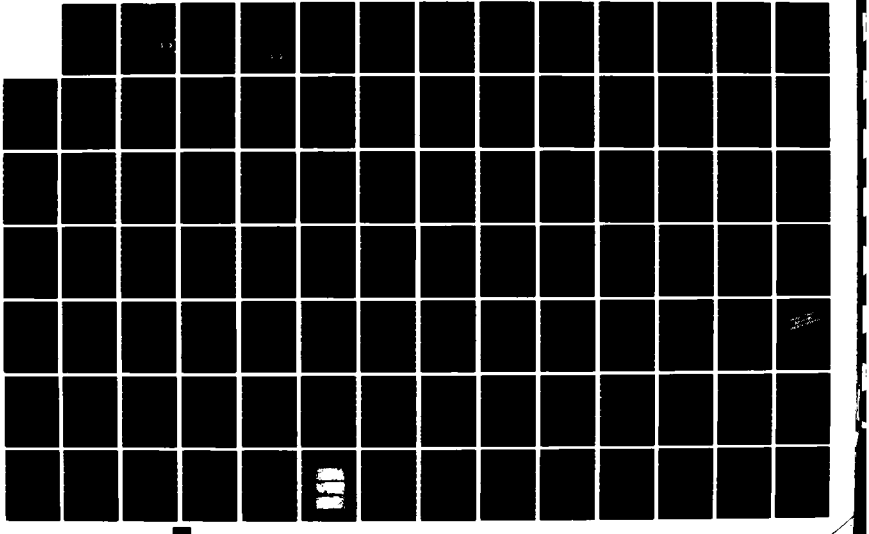

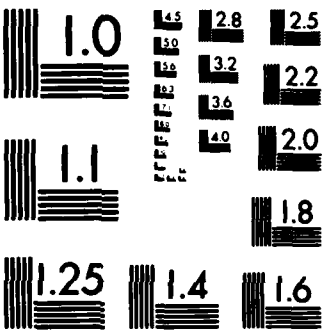

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Final Report

CHEMICAL CARCINOGEN-INDUCED CHANGES IN tRNA METABOLISM IN HUMAN CELLS

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Department of Physiological Chemistry

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

It was proposed that changes in tRNA metabolism are required for cells to progress through the stages of carcinogenesis, and a comprehensive hypothesis was formulated to describe tRNA-mediated endogenous promotion of carcinogenesis. This hypothesis offers a viable explanation for the lengthy time frame observed between carcinogen exposure and neoplastic transformation. A role was defined for 7-methylguanine as an endogenous promoting agent, whereby this natural RNA catabolite induces queuine hypomodification in the tRNA anticodon by inhibiting the queuine insertion enzyme tRNA-guanine ribosyltransferase. Subsequently, 7-methylguanine induces neoplastic transformation.

A cell culture system was developed which allows the study of tumor promoter-induced mimicry of transformation with normal human cells, and using this system, phorbol ester tumor promoters were also demonstrated to induce queuine hypomodification of tRNA. However, in this case, the hypomodification occurred due to a specific phorbol ester

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18. (cont.)
ribosyltransferase.

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inhibition of queuine transport into the cells. Most importantly, overcoming the tumor promoter-induced hypomodification of tRNA by supplying the cells with excess queuine, blocked the expression of a transformed phenotype by the human cells. Therefore, queuine may be an anti-promoting compound, and a role for queuine hypomodification in the expression (promotion) of carcinogenesis appears likely.

Finally, a previously undescribed tRNA modification enzyme was discovered; an enzyme involved in the biosynthesis of inosine in the first (wobble) position of the anticodon of selected tRNAs. This hypoxanthine insertion enzyme, a tRNA-hypoxanthine ribosyltransferase, may have an important role in regulating protein synthesis, since inosine in the wobble position greatly expands the mRNA codon recognition potential of that tRNA. Evidence was obtained that the hypoxanthine insertion reaction may be involved in cell differentiation, and that defects in insertion may be involved in the dedifferentiation associated with neoplasia. Again, the results are consistent with the model for tRNA-mediated promotion of carcinogenesis.

B. Research Objectives

The grant proposal submitted in 1980 tendered the hypothesis that changes in tRNA metabolism are required for the progression of cells through the stages of carcinogenesis. Following the initiation of the carcinogenic process, gradual phenotypic modulation towards a less differentiated cellular state occurs. Alterations in tRNA modification and catabolism are known to commence soon after chemical carcinogen exposure, and the accruing changes in tRNA isoaccepting species were hypothesized to be involved in releasing post-transcriptional controls over developmental gene expression.

At some early time after the initiation of carcinogenesis there is an elevation of tRNA methylation which leads to an increase in the levels of methylated RNA catabolites. These catabolites may modulate tRNA modification further, either by inhibiting anticodon modifications by tRNA ribosyltransferases or by acting as alternate substrates for these enzymes. New tRNA isoaccepting species are generated by the changes in macromolecular modification and these species should translate different (e.g. fetal) mRNAs that are present. By this method, onco-developmental proteins would be synthesized. Some of the new proteins could be tRNA methyltransferases, and this would start the cycle over again at a more aberrant level. After many such cycles, the stage of neoplastic transformation could be reached. We have examined these variations in tRNA metabolism to determine if they are, in fact, involved in the dedifferentiation and progression of carcinogenesis.

C. Status of the Research

We have been interested for some time in the role of tRNAs in various cellular regulatory processes. This family of highly complex macromolecules carries out the pivotal role in translating the genetic code from a sequence of nucleotides to a sequence of amino acids, i.e., the tRNAs carry the appropriate amino acids and read the triplet codes specified in mRNAs. The correct sequences of amino acids are then linked together during protein synthesis.

All tRNAs have the general two-dimensional (2-D) cloverleaf structure depicted in Figure 1. According to this scheme, the amino acid is linked to an adenosine moiety at position 76, and the anticodon responsible for interacting with the codon in mRNA is specified by positions 34, 35, and 36. While all tRNAs have the same general 2-D structure (and probably similar 3-D structures as well), the individual tRNAs must be sufficiently unique to insure the fidelity of both aminoacylation and codon recognition, i.e., the tRNA must be matched specifically with the correct amino acid and the correct codon to preclude errors in protein synthesis. Perhaps it is for this reason that structurally, tRNAs are the most complex class of biological macromolecules known to exist.

Of the 73 to 93 nucleosides present in the primary sequence of the mature, cytosol tRNAs (Figure 1), approximately 10-15 are modified in one way or another in eukaryotic cells. Over 50 different modified nucleosides have been identified in tRNA macromolecules, and different sites in the primary sequence are modified in different tRNA species, thereby making each unique. The tRNA

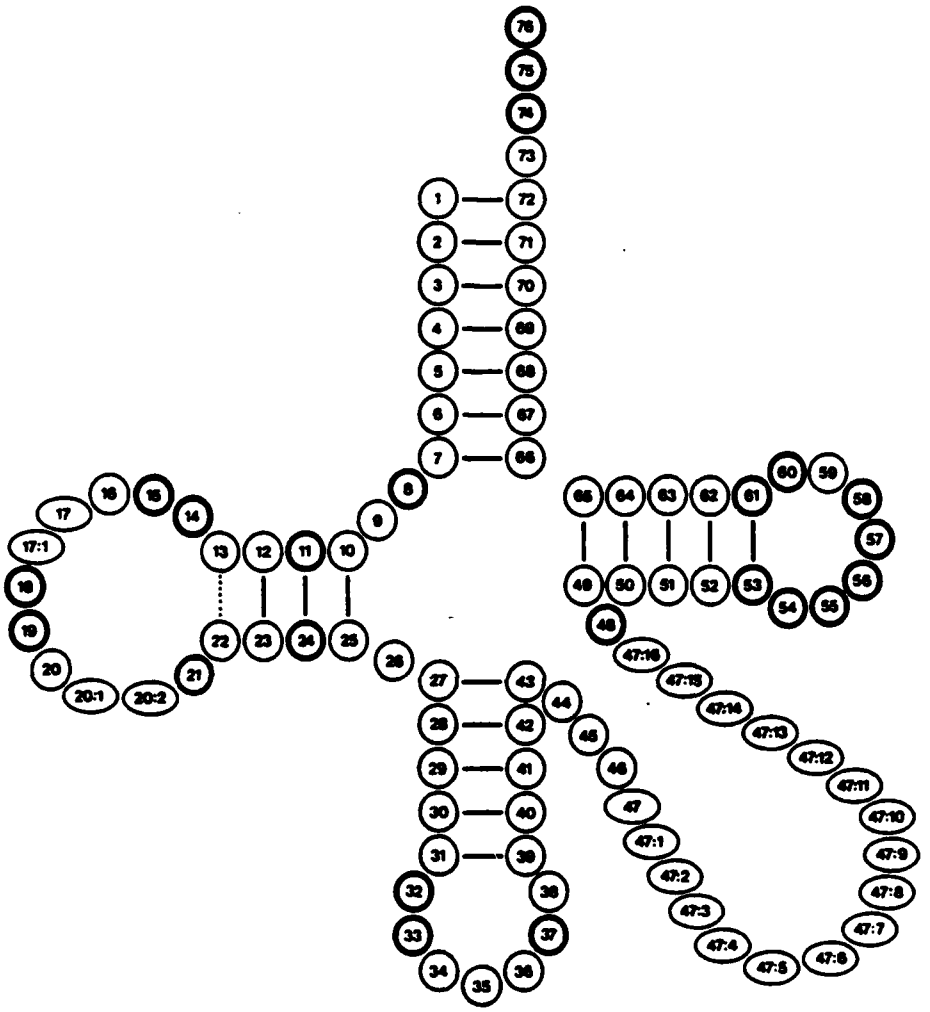


Figure 1. Uniform numbering system for the nucleosides in tRNAs. The numbering scheme was proposed at a Cold Spring Harbor tRNA meeting in 1978, and it is based on yeast $tRNA^{Phe}$. Typically, extra nucleotides are found in other tRNAs after positions 17, 20, and 47, and therefore, the designations 17:1, 20:1, 47:1, etc. The total number of nucleotides in tRNAs sequenced to date range from 73 to 93. The heavy circles depict the positions of nucleosides that are common to many tRNAs. For example, positions 74, 75, and 76 represent the $-C-C-A_{OH}$ 3' terminus common to all functional tRNAs. The anticodon is represented by positions 34, 35, and 36.

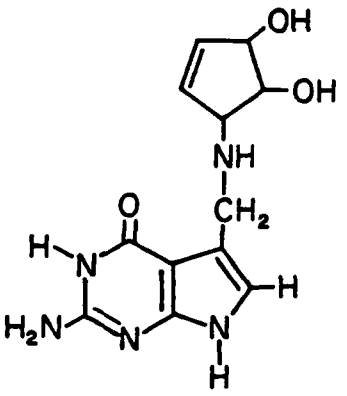
modifications occur at the macromolecular level (after the primary RNA transcript has been specified), and it may take upwards of 200 different enzymes to carry out these very specific modification reactions.

The modified nucleosides and bases found in tRNAs may be relatively simple or highly complex. The purine derivatives depicted in Figure 2 illustrate the extremes in structural variation that can be found. While hypoxanthine is merely a deaminated form of the major base adenine (although, as will be described later, it does not arise from a macromolecular deamination reaction), queuine is an extremely modified guanine analog. Both queuine and hypoxanthine are found exclusively in the first position of the anticodon (position 34) of the tRNAs indicated in Figure 2. Therefore, they are located in the area of the macromolecule that is involved in codon recognition, and so, they have the potential to influence gene expression.

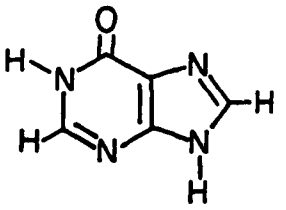
Most tRNA modification reactions occur by the addition of some moiety (e.g. the methyl group from S-adenosylmethionine) to one of the four major nucleosides (adenosine, guanosine, cytidine, and uridine) found in the primary transcript. However, the enzyme involved in generating the queuine-containing tRNAs, tRNA-guanine ribosyltransferase (EC 2.4.2.29), utilizes a different mechanism (Figure 3). This modification occurs by a direct base replacement of queuine for guanine only in the first position of the anticodon of tRNA isoacceptors for aspartic acid, asparagine, histidine, and tyrosine. Until recently, this was the only tRNA modification reaction known to occur by a direct base replacement.

Mammalian cells obtain queuine from the diet or gut flora in vivo and from sera utilized to supplement cell culture media in vitro. Although queuine is a dietary factor that is not synthesized by mammalian cells, normal mature tRNAs are usually fully queuine modified, i.e., they are in the $tRNA_Q$ form depicted in Figure 3. However, when mammalian cells undergo neoplastic transformation in vivo or in vitro, one of the most common tRNA structural changes involves a decrease in queuine modification. Under these circumstances, one or more of the isoacceptors for aspartic acid, asparagine, histidine, and/or tyrosine may have guanine instead of queuine in the first position of the anticodon, i.e., they are in the $tRNA_G$ form depicted in Figure 3. This decrease in queuine-containing tRNA in transformed cells could arise from substrate limitation or enzyme inhibition, but the functional significance of the altered queuine content in the tRNA anticodon has not been established. However, the queuine hypomodified tRNAs are also found in immature, undifferentiated cells, so they may be considered to be onco-developmental tRNAs.

While a specific role for queuine hypomodified tRNA in carcinogenesis was still a subject of conjecture, it represented a central component of our hypothesis formulated for tRNA-mediated endogenous promotion of carcinogenesis. The sequence of events in altered tRNA metabolism as it is visualized to occur in carcinogenesis is presented in Figure 4. At some early time after the initiation of carcinogenesis, there is an elevation of enzymatic RNA methylation which leads to the increase in the levels of methylated RNA catabolites. These catabolites may modulate tRNA modification further, either by inhibiting modification (e.g. by blocking tRNA-guanine ribosyltransferase) or by acting as an alternative substrate. New tRNA isoaccepting species are generated by the



queuine



hypoxanthine

$tRNA^{Asp}$ $tRNA^{His}$
$tRNA^{Asn}$ $tRNA^{Tyr}$

$tRNA^{Ala}$ $tRNA^{Pro}$
$tRNA^{Arg}$ $tRNA^{Ser}$
$tRNA^{Ile}$ $tRNA^{Thr}$
$tRNA^{Leu}$ $tRNA^{Val}$

Figure 2. The structures of queuine and hypoxanthine. These modified purines have been found only in the first position of the anticodon (position 34 in Figure 1) of the tRNAs indicated.

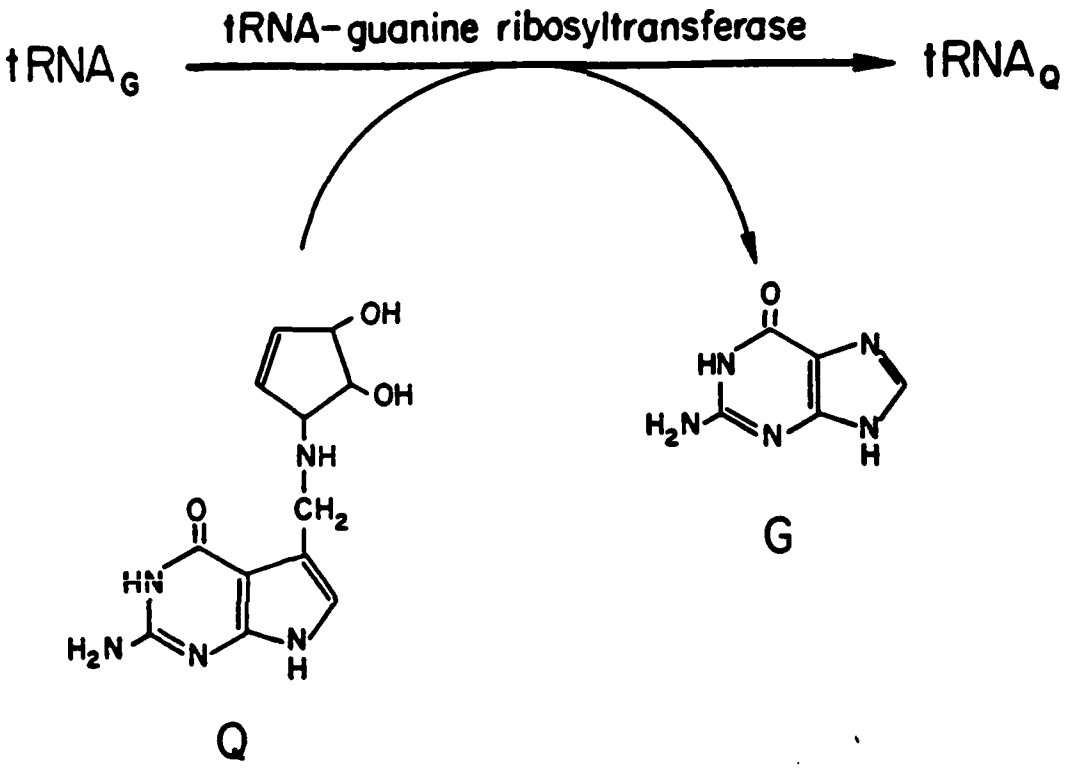


Figure 3. tRNA-guanine ribosyltransferase reaction. The enzyme exchanges queuine for guanine in the first position of the anticodon of mammalian tRNAs for aspartic acid, asparagine, histidine, and tyrosine. The abbreviations are: Q, queuine; and G, guanine.

TRANSFER RNA MEDIATED ENDOGENOUS PROMOTION OF CARCINOGENESIS

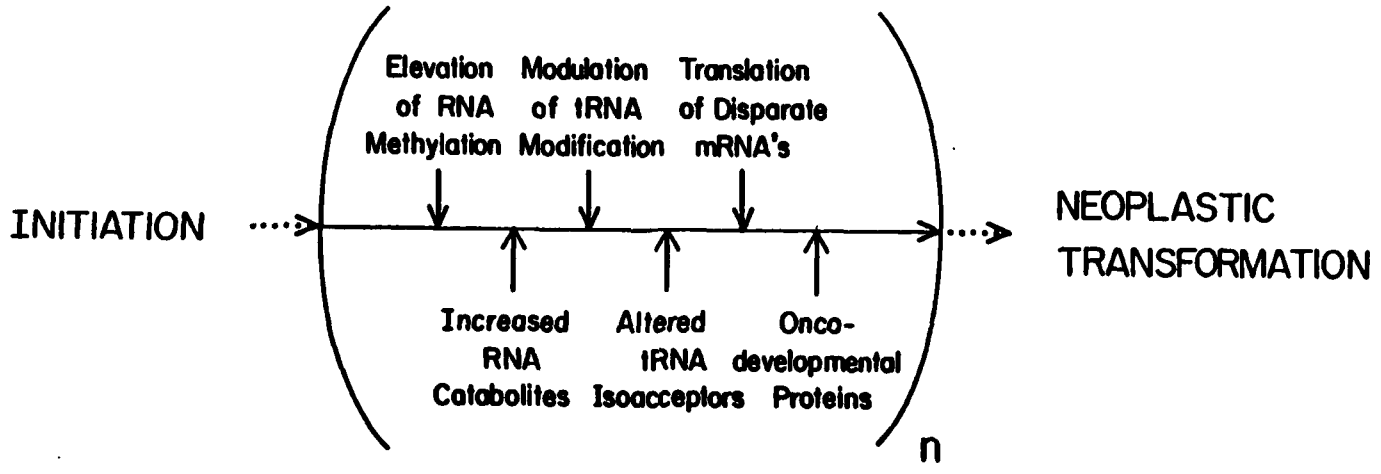


Figure 4. Proposed model for tRNA-mediated endogenous promotion of carcinogenesis. The initiation event could be by any means, but it likely involves a heritable change in DNA. The sequence of events depicted is then predicted to occur in order with each being caused by the previous event. Soon after initiation there would be an increase in nucleic acid (mainly tRNA) methyltransferase activity which would result in an increase in methylated nucleic acid catabolites. The higher endogenous levels of methylated purines (e.g. 7-methylguanine) would then modulate tRNA modification by inhibiting tRNA ribosyltransferases. The methylated purines might also modulate tRNA modification by acting as alternate substrates for the ribosyltransferases or by other, as yet unidentified, means. Both the modulation and methylation steps depicted could be involved in generating altered tRNA isoaccepting species, and these onco-developmental species are predicted to efficiently translate existing (previously poorly translated) mRNAs. It is assumed that some of these translation products would be onco-developmental proteins. If any of these proteins were onco-developmental tRNA methyltransferases, the cycle would repeat at a more aberrant level. Phenotypic alterations could continue to accrue by further cycling (staging) until neoplastic transformation is attained. Also see Figure 5.

changes in macromolecular modification, and these species might translate different (e.g. onco-developmental) mRNAs that are present but are not translated efficiently by the normal tRNAs. By this method, onco-developmental proteins would be synthesized. Some of the new proteins could be tRNA methyltransferases, and this would start the cycle over again at a more aberrant level (Figure 5). Finally, after many such cycles, the stage of neoplastic transformation would be reached. By this means, one can explain the lengthy time frame involved in the neoplastic process subsequent to the initiation event in vivo or in vitro (Figure 5). [See Trewyn et al., 1983 (appended) for additional details].

It should be pointed out that each of the individual changes depicted in Figure 4 (e.g. modulation of tRNA modification, altered tRNA isoacceptors, translation of disparate mRNAs, etc.) are all relatively well documented alterations when one compares normal cells to malignant cells of the same type (Trewyn et al., 1983). What is unique about the hypothesis outlined in Figure 4 is the assignment of cause and effect relationships between the individual changes, and more importantly, the designation of a fundamental role for these changes in the expression (promotion) of carcinogenesis.

Our recent studies suggest that the pivotal point in the hypothesized model for tRNA-mediated endogenous promotion of carcinogenesis involves modulation of tRNA modification. We demonstrated previously that exogenous methylated purines (e.g. 7-methylguanine) can transform normal Chinese hamster cells in vitro. We then discovered that 7-methylguanine inhibited tRNA-guanine ribosyltransferase, thereby inducing queuine hypomodification, under the conditions leading to neoplastic transformation [Elliott and Trewyn, 1982 (appended); Trewyn et al., 1983 (appended)]. Therefore, this normal RNA catabolite (7-methylguanine) was able to modulate tRNA modification as postulated in Figure 4. Since significant increases in the generation of endogenous methylated purines (including 7-methylguanine) occur very early during tumor development in vivo, it is likely that the addition of exogenous 7-methylguanine in vitro was mimicing the situation in vivo.

Our studies with human diploid fibroblasts in vitro demonstrated no significant effects of exogenous 7-methylguanine on inducing transformation or queuine hypomodification of tRNA (unpublished data). Of course, that does not mean that endogenous RNA catabolites would not modulate tRNA modification as hypothesized. Because the methylated purines appeared to be acting as promoting agents (as opposed to inducing agents) in the Chinese hamster cell model system (Trewyn et al., 1983), phorbol ester tumor promoters were evaluated for their effects on human fibroblasts. Phorbol esters elicit numerous changes in the growth characteristics of cultured rodent cells even in the absence of initiation of carcinogenesis; an in vitro phenomenon referred to as mimicry of transformation. However, there had been no reports of these agents inducing the expression of transformed phenotypes with normal human cells in vitro under standard culture conditions.

We discovered that the mouse skin tumor promoter phorbol 12,13-didecanoate (PDD) will induce significant increases in the saturation density of normal human cells cultured in medium containing 8X nonessential amino acids [Trewyn and Gatz, 1984 (appended)]. The saturation density for the normal control cells

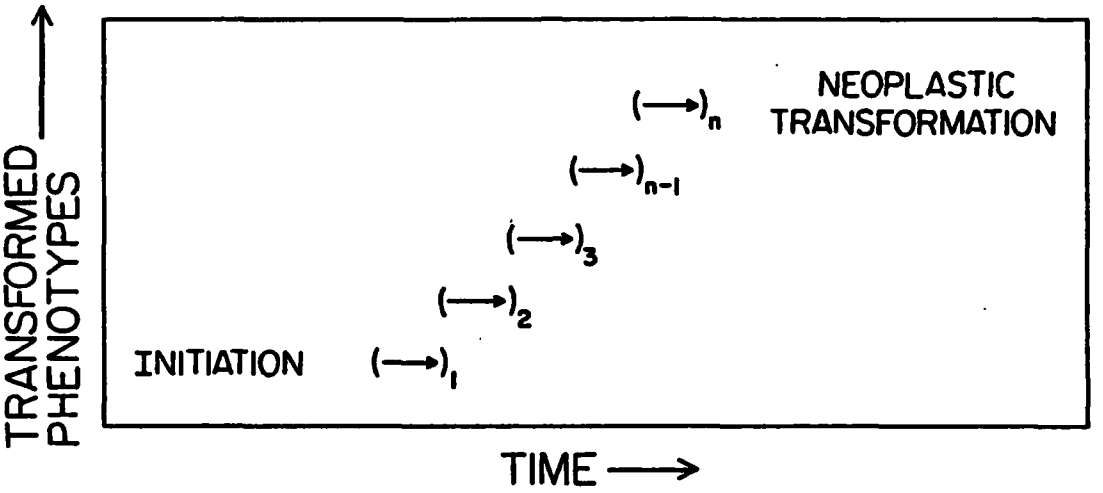


Figure 5. Cycling (staging) phenomenon giving rise to the endogenous promotion of transformed phenotypes. With time, the cycle depicted in Figure 4 would repeat many times with increasing expression of transformed phenotypes being the result until neoplastic transformation is attained.

was approximately 4 x 10^4 cells/cm^2 at most passages, while the PDD treated cells maintained levels 3 to 4-fold higher for most of the lifetime of the cultures. The specificity of the effect for an active tumor promoter was demonstrated by the fact that the inactive analog 4α-phorbol 12,13-didecanoate (4α-PDD) gave results similar to the untreated controls. It should also be noted that the same culture medium (containing 8X nonessential amino acids) enhances the selection of initiated cells following exposure to chemical carcinogens [Milo and Trewyn, 1982 (appended)].

Because aspartic acid and asparagine (2 of the 4 amino acids normally using queuine modified tRNAs) are nonessential amino acids, we next examined combinations of amino acids which included all 4 of those for the queuine tRNAs. It was postulated that the hypomodified tRNAs might be aminoacylated less efficiently, and therefore, higher concentrations of the amino acids would be required for these tRNAs to participate in protein synthesis. We discovered an even greater initial increase in the saturation density of normal cells when they were treated with PDD in medium supplemented with 2X aspartic acid, asparagine, histidine, tyrosine (those for the queuine tRNAs; some of which are hypomodified in transformed cells) and phenylalanine (its tRNA is usually hypomodified for wye-base in transformed cells). Cell densities in the PDD treated cultures reached levels 5 to 10-times those in the untreated and 4α-PDD treated controls. Similar results have been obtained with many different primary cultures in this medium. Although the timing of this transient increase in density has been somewhat variable, all the cultures exhibited a sustained 2 to 4-fold elevation subsequently, similar to that seen in 8X nonessential amino acids (Trewyn and Gatz, 1984). Other suspected tumor promoters (e.g. mezerein and 12-O-tetradecanoylphorbol-13-acetate) induced analogous phenotypic changes suggesting the usefulness of this model system for evaluating human cancer promoting agents [Davakis and Trewyn, 1983 (appended)].

As described previously (Elliott and Trewyn, 1982; Trewyn et al., 1983), 7-methylguanine induces queuine hypomodification of tRNA and promotes the expression of transformed phenotypes in normal Chinese hamster cells. We now have evidence that phorbol ester tumor promoters elicit similar tRNA changes in normal human cells [Elliott et al., 1984 (appended)]. Significant queuine hypomodification of tRNA was observed in human cells treated continuously with PDD, and this hypomodification preceded the transient increase in saturation density promoted by PDD (Figure 6). The timing of both the induced queuine hypomodification and the increase in saturation density have been somewhat variable from one primary culture to another, but in numerous independent experiments, maximal queuine hypomodification preceded the maximal saturation density as in the experiment depicted (Figure 6). In all experiments, a reversal of the queuine hypomodification then preceded a decrease in saturation density. The inactive tumor promoter 4α-PDD had a minimal effect on queuine hypomodification and saturation density.

The transient queuine hypomodification of tRNA indicated by the enzymatic assay using E. coli tRNA-guanine ribosyltransferase (Figure 6) was verified by RPC-5 chromatography of $tRNA^{His}$ isoacceptors (Figure 7). As can be seen, PDD induced a major shift from the queuine-modified species to the queuine-deficient species. Interestingly, PDD did not cause a similar shift in tRNA isoacceptors for aspartic acid (data not presented). The other two queuine-containing tRNAs ($tRNA^{Asn}$ and $tRNA^{Tyr}$) were not evaluated.

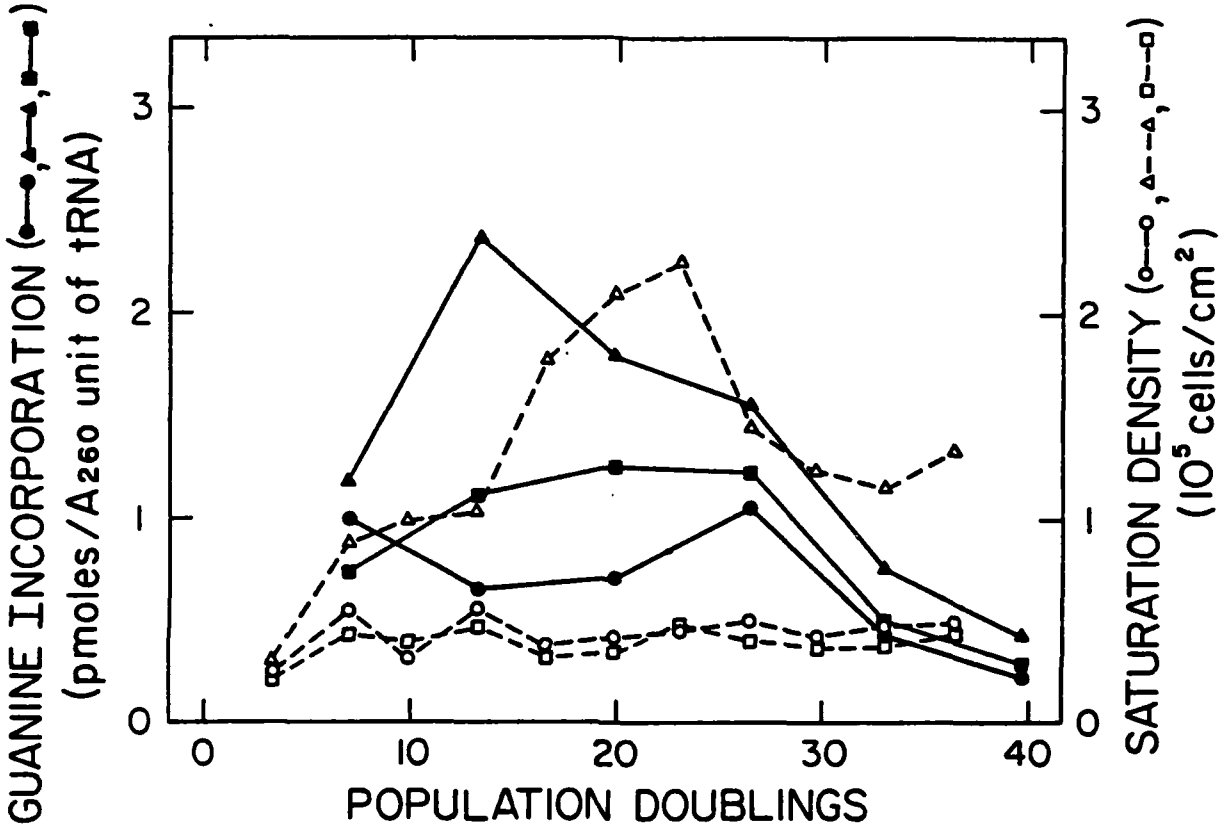


Figure 6. Decreased queuine modification of tRNA in human cells induced by PDD. The tRNA from control cells (●) and cells treated with 10^{-7}M PDD (▲) or 4α-PDD (■) was assayed for increased guanine incorporation (decreased queuine modification) at increasing cell population doublings in culture. The saturation densities of the control culture (0) and the PDD (Δ) and 4α-PDD (□) treated cultures are also indicated. See Elliott et al. [1984 (appended)] for additional details.

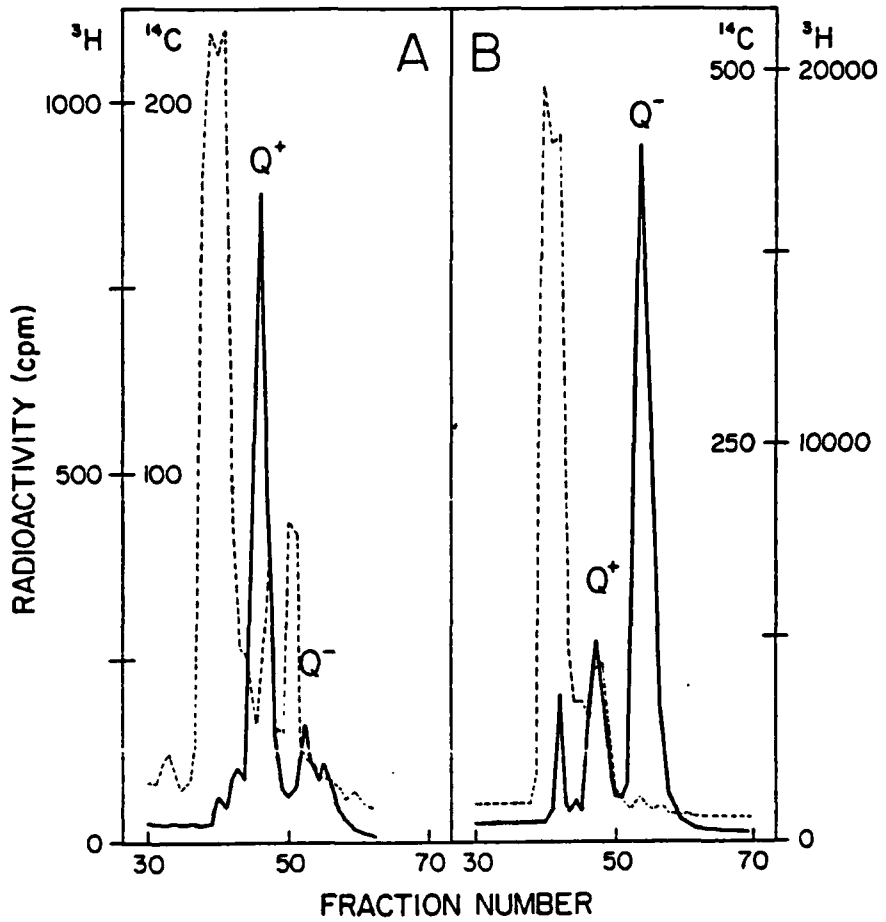


Figure 7. RPC-5 chromatographic profiles of $tRNA^{His}$. The solid lines represent: (A), [^{14}C]histidine-tRNA derived from second passage human skin cells; and (B), [^{14}C]histidine-tRNA derived from second passage human skin cells treated with 10^{-8}M PDD. Major human fibroblast elution peaks are designated as the Q^+ ($tRNA_Q$) and Q^- ($tRNA_G$) isoacceptors. Dotted lines represent [^{3}H]histidine-tRNA derived from normal mouse liver. Purified tRNA from both PDD-treated and untreated human skin cells was aminoacylated with radiolabelled histidine using a mouse liver derived aminoacyl-tRNA synthetase preparation. The histidyl-tRNA isoacceptors were separated on a column (0.9 x 20 cm) of RPC-5 support developed at 27°C with a 150 ml 0.475 to 0.80 M NaCl linear gradient in 10 mM sodium acetate buffer (pH 4.5), 10 mM $MgCl_2$, 3 mM 2-mercaptoethanol and 1 mM EDTA; the flow rate was 1.4 ml/min, 1.5 ml fractions were collected, and the radioactivity was measured by liquid scintillation counting. See Elliott et al. [1984 (appended)] for additional details.

Based on the molecular mechanism for queuine modification of tRNA (Figure 3), the transient nature of the hypomodification induced in the continuous presence of PDD was unexpected. However, it was discovered that newly established cultures of human skin fibroblasts lack the ability to salvage endogenous queuine from tRNA turnover, whereas later passage cells acquire this ability (Elliott et al., 1984). Again, the timing of induction of maximal queuine salvage from tRNA turnover was somewhat variable from one primary culture to another, but it was usually attained by 15 to 20 population doublings (4 to 6 passages) in culture. Salvage allows the reuse of queuine derived from tRNA catabolism, thereby decreasing the exogenous queuine requirement of the cells.

The serum utilized to supplement the cell culture medium contains queuine, and this is the source of queuine for tRNA modification in cultured cells. Our data indicated, however, that a PDD-induced, queuine limitation in the cells had some role in the major increase in saturation density. Therefore, excess queuine (5×10^{-8}M) was added to cells treated with PDD (10^{-8}M), and saturation densities were monitored (Figure 8). The excess queuine significantly reduced the increase in saturation density induced by PDD, while having little effect on saturation densities in the absence of PDD. The data demonstrate that the expression of a transformed phenotype can be modulated by the tRNA-guanine ribosyltransferase substrate queuine.

While 7-methylguanine generated queuine-deficient tRNA in Chinese hamster cells by inhibiting tRNA-guanine ribosyltransferase (Elliott and Trewyn, 1982), PDD only marginally inhibited tRNA-guanine ribosyltransferase in vitro (data not presented). However, PDD can induce queuine hypomodified tRNA in normal human fibroblasts by blocking uptake of exogenous queuine (Figure 9). 7-Methylguanine and 4-O-methyl-TPA had some effect on diminishing queuine transport (Figure 9), but not nearly so much as PDD. The queuine transporter appears to be highly efficient with a K_m of approximately 3×10^{-8}M [Elliott, et al., in press (appended)]. Therefore, excess exogenous queuine must overcome the PDD-induced inhibition of queuine transport, and thereby allow the tRNA anticodon modification to occur. The dietary component queuine might thus be a useful biologic response modifier.

As mentioned earlier, the only tRNA modification reaction previously known to occur by a base exchange mechanism was the one catalyzed by tRNA-guanine ribosyltransferase (Figure 3). However, we felt that additional reactions of this type might be involved in anticodon modifications in other tRNAs; a possibility that could have important ramifications for our postulated model dealing with promotion of carcinogenesis (Figures 4 and 5).

Our investigations into tRNA modifications within the anticodon region have led to the identification of an enzymatic activity which incorporates hypoxanthine (Figure 2) into mature tRNA macromolecules [Elliott and Trewyn, 1984 (appended)]. This enzyme is postulated to be similar to tRNA-guanine ribosyltransferase. The previously undocumented hypoxanthine incorporating enzyme has been assayed in rat liver and human leukemia cell cytosolic preparations, and it was resolved from tRNA-guanine ribosyltransferase by DEAE-cellulose column chromatography (Figure 10). Whereas tRNA-guanine

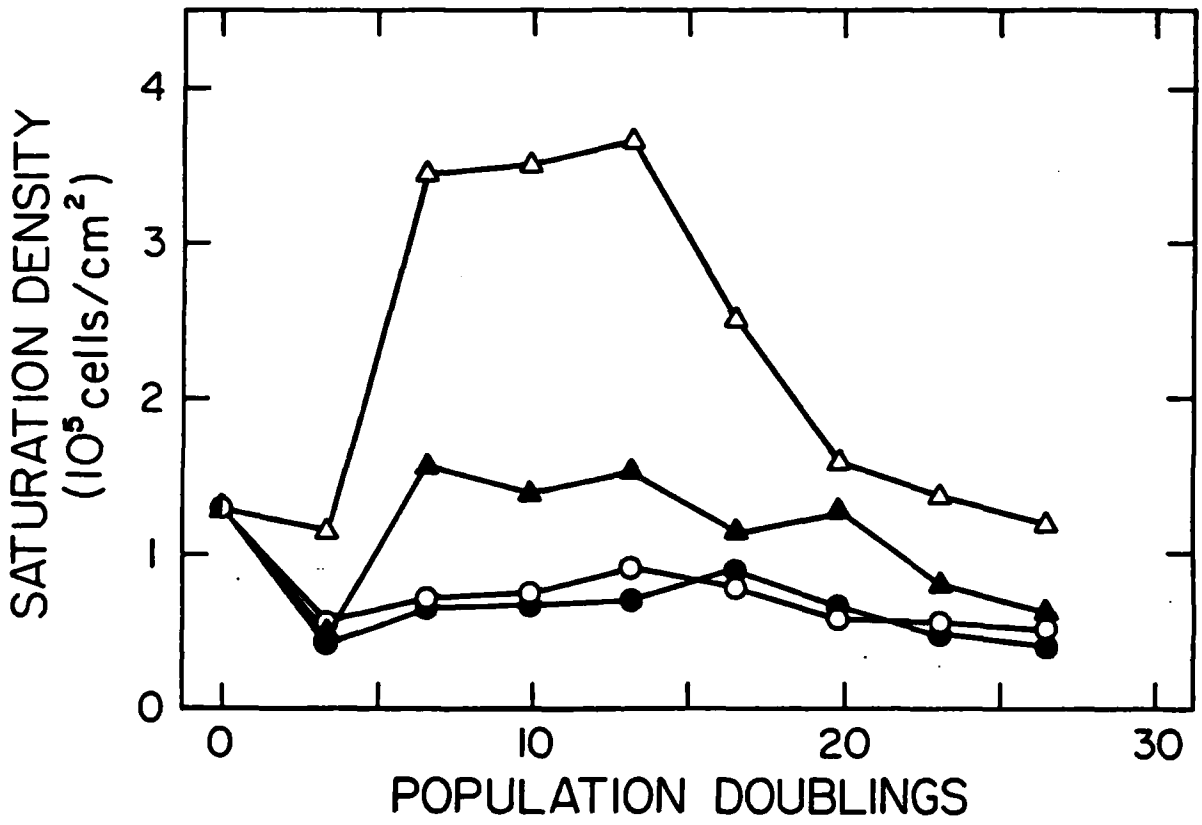


Figure 8. Saturation densities of human cells exposed continuously to PDD with or without excess queuine. The cell populations are: Control (O), control plus 5×10^{-8} M queuine (●), 10^{-8} M PDD (Δ), and 10^{-8} M PDD plus 5×10^{-8} M queuine (▲). See Elliott et al. [submitted (appended)] for additional details.

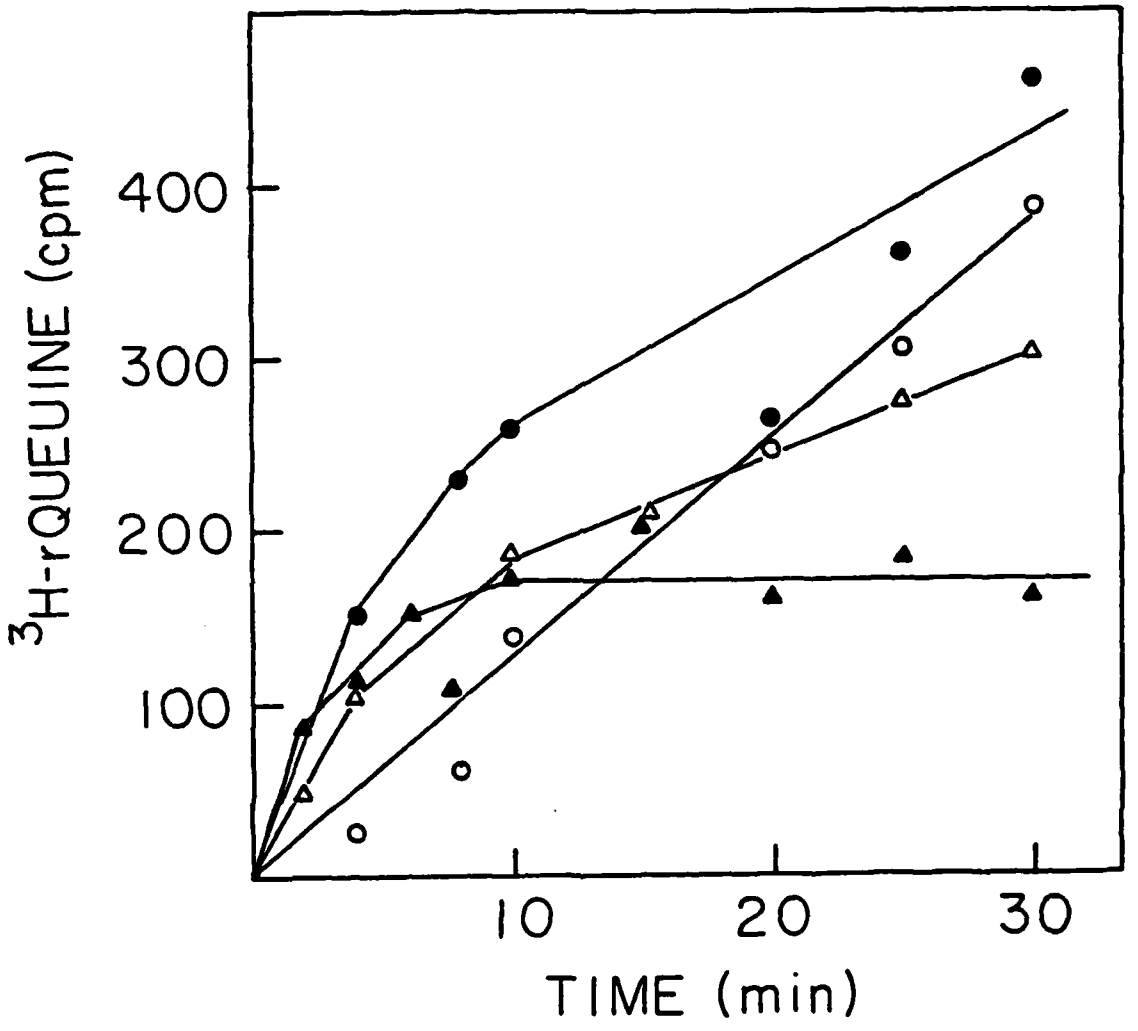


Figure 9. Inhibition of [^{3}H]dihydroqueuine transport into human skin fibroblasts by PDD. Post-confluent, early passage human fibroblasts in culture medium supplemented with 10% queuine-deficient fetal bovine serum (Katze, 1978) were fed with the same fresh medium alone (●) or containing 10^{-8}M PDD (▲), 10^{-5}M 7-methylguanine (○), or 10^{-8}M 4-O-methyl-TPA (△). The cells were incubated for 20 minutes at 37°C and then 500 nM [^{3}H]dihydroqueuine (0.12 Ci/mmole) was added. Uptake was monitored in duplicate cultures for 0 to 30 minutes. Transport of [^{3}H]dihydroqueuine was stopped by decanting the medium and rinsing the cells 3 times with ice-cold PBS. The cells were lysed with 0.5 ml of 95% ethanol, and the lysate was counted by liquid scintillation.

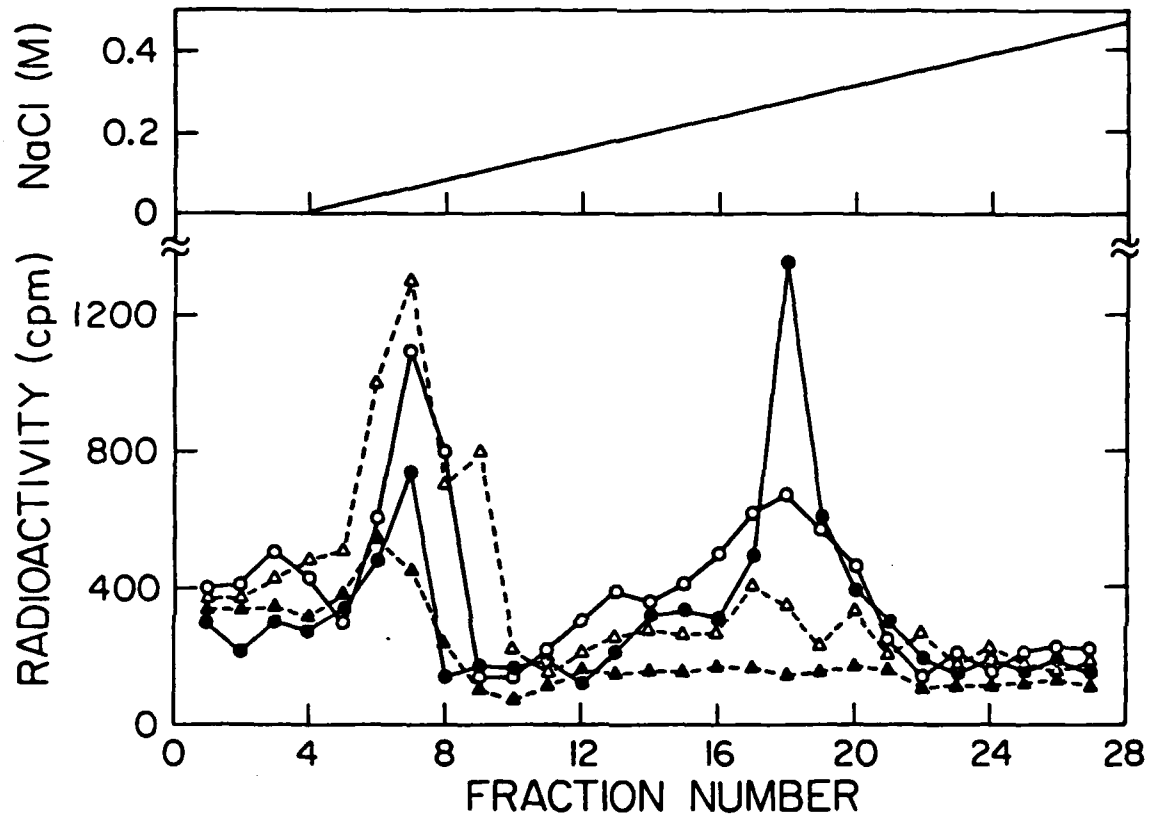


Figure 10. Chromatography of rat liver tRNA ribosyltransferases on a DEAE-cellulose column. The assay conditions were similar to those described in the Methods section. The symbols indicate; E. coli tRNA and [8-^{3}H]hypoxanthine (●); yeast tRNA and [8-^{3}H]hypoxanthine (○); E. coli tRNA and [8-^{3}H]guanine (▲); and yeast tRNA and [8-^{3}H]guanine (△). See Elliott and Trewyn [1984 (appended)] for additional details.

ribosyltransferase (peak I) has substrate preferences for guanine and yeast tRNA in vitro, the putative tRNA-hypoxanthine ribosyltransferase (peak II) utilizes hypoxanthine and E. coli tRNA much more readily. The vast differences in substrate utilization and in column elution characteristics indicate that unique enzyme activities were being assayed.

The tRNA-hypoxanthine ribosyltransferase assay is based on the incorporation of radiolabelled hypoxanthine into unfractionated tRNA from various sources. The reaction rate is linear for at least the first 30 to 40 minutes, after which a plateau is reached when no additional hypoxanthine can be incorporated. The tRNA-hypoxanthine ribosyltransferase reaction rate is proportional to the amount of substrate E. coli tRNA, as well as the amount of added enzyme extract. E. coli tRNA is a better substrate than yeast tRNA for both the rat liver and human leukemia cell enzymes. This correlates inversely with the amount of inosine (the nucleoside of hypoxanthine) found by HPLC analyses of the tRNA hydrolysates, i.e., substrate E. coli tRNA contains less inosine than yeast tRNA prior to the in vitro enzyme assay. Most importantly, subsequent to the in vitro assay, radiolabelled inosine was the predominant product found in tRNA hydrolysates (Figure 11), thereby demonstrating the covalent insertion of hypoxanthine.

Kinetic analyses of hypoxanthine incorporation into unfractionated E. coli tRNA yielded a K_m of approximately 5 x 10^{-6}M for hypoxanthine using a crude cell extract from cultured human leukemia cells (Figure 12). Adenine was a competitive inhibitor of the hypoxanthine insertion reaction, with a K_i of approximately 2 X 10^{-6}M (Figure 12). None of the other major bases (guanine, cytosine, uracil, or thymine) inhibited the reaction, and hypoxanthine, adenine, guanine, cytosine, and uracil were all incapable of exchanging the radiolabeled hypoxanthine out of the E. coli tRNA, indicating that the covalent hypoxanthine insertion is irreversible.

While the identity of the base in the primary transcript being exchanged for hypoxanthine remains to be established definitively, it is likely that adenine (adenosine) is converted to hypoxanthine (inosine). Recent data (Kretz and Trewyn, unpublished) suggest that adenine is not only an inhibitor of hypoxanthine incorporation into the tRNA, but that it is a substrate as well (Figure 13). Therefore, it appears likely that the tRNA-hypoxanthine ribosyltransferase can catalyze an adenine for adenine exchange reaction just as the queuine insertion enzyme can catalyze a guanine for guanine exchange. However, if the preferred substrate is available (hypoxanthine for tRNA-hypoxanthine ribosyltransferase and queuine for tRNA-guanine ribosyltransferase) an essentially irreversible exchange occurs. The hypoxanthine insertion reaction responsible for inosine biosynthesis in tRNA, as it is visualized to occur, is depicted in Figure 14.

Inosine is found in tRNA only in the first position of the anticodon, and in that position, it has the potential to influence gene expression significantly. According to the wobble hypothesis of codon-anticodon pairing proposed by Francis Crick, inosine in the first position of the anticodon of tRNAs could base-pair with uridine, cytidine, or adenosine in the third position of appropriate codons in mRNA (Table 1), while standard base-pair interactions would be maintained in the other two positions. Adenosine in the tRNA wobble position would base-pair only with uridine, guanosine with cytidine or uridine,

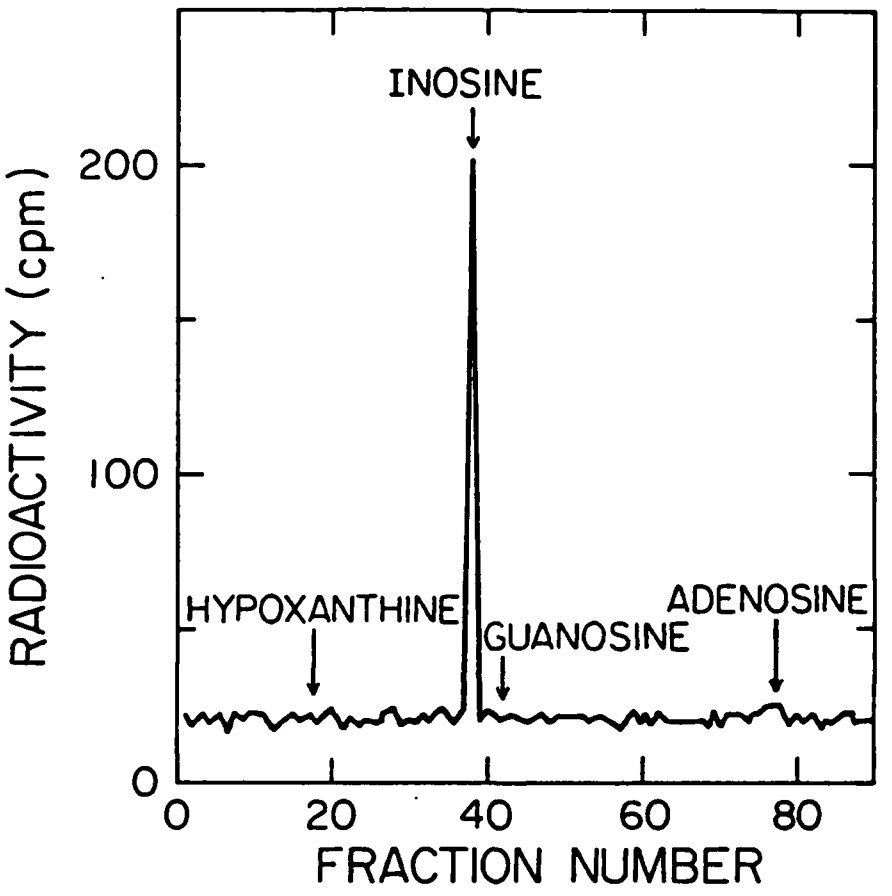


Figure 11. HPLC elution profile of hydrolyzed E. coli tRNA radiolabelled with [8-^{3}H]hypoxanthine. Cultured HL-60 cells were used as a source of the enzyme. The enzyme assay, isolation and hydrolysis of the tRNA, and chromatography conditions were as described in the Methods section. The elution positions of authentic purine nucleosides and hypoxanthine are indicated. See Elliott and Trewyn [1984 (appended)] for additional details.

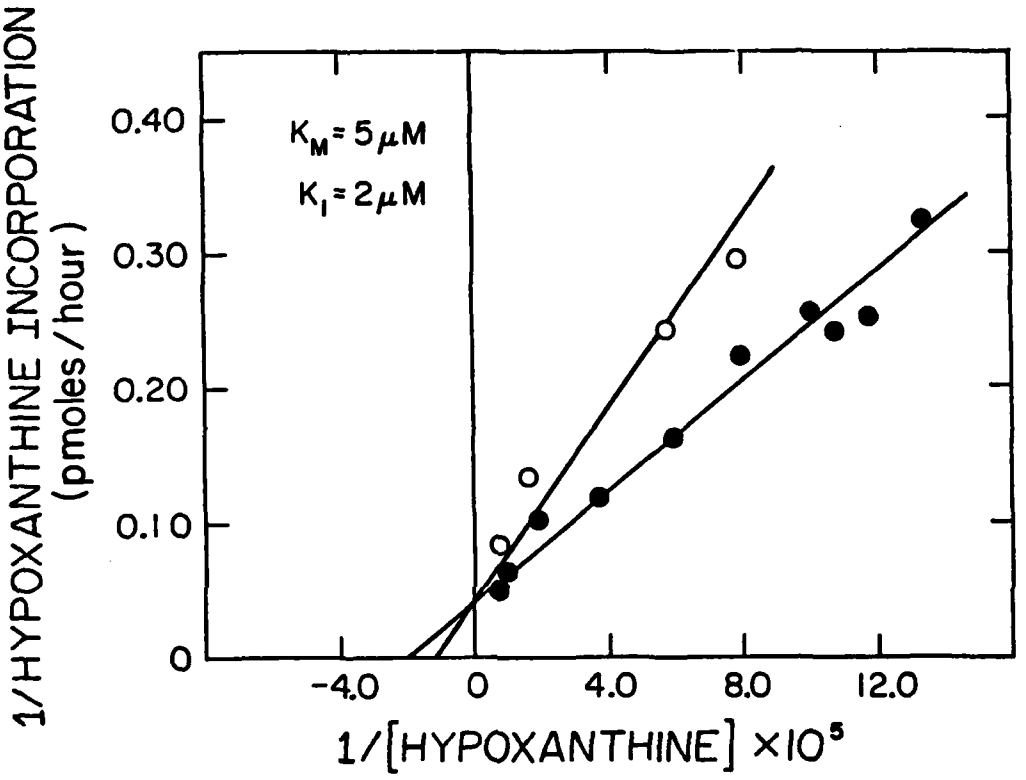


Figure 12. Lineweaver-Burk analysis for tRNA-hypoxanthine ribosyltransferase. Cultured human malignant T-lymphoblasts were used as a source of the enzyme. The assay conditions are described in Elliott and Trewyn [1984 (appended)], with hypoxanthine concentrations from 0.75-15.75 μM. The mean of triplicate assays is indicated at each point for control reactions (●) and those containing 10 μM adenine (○).

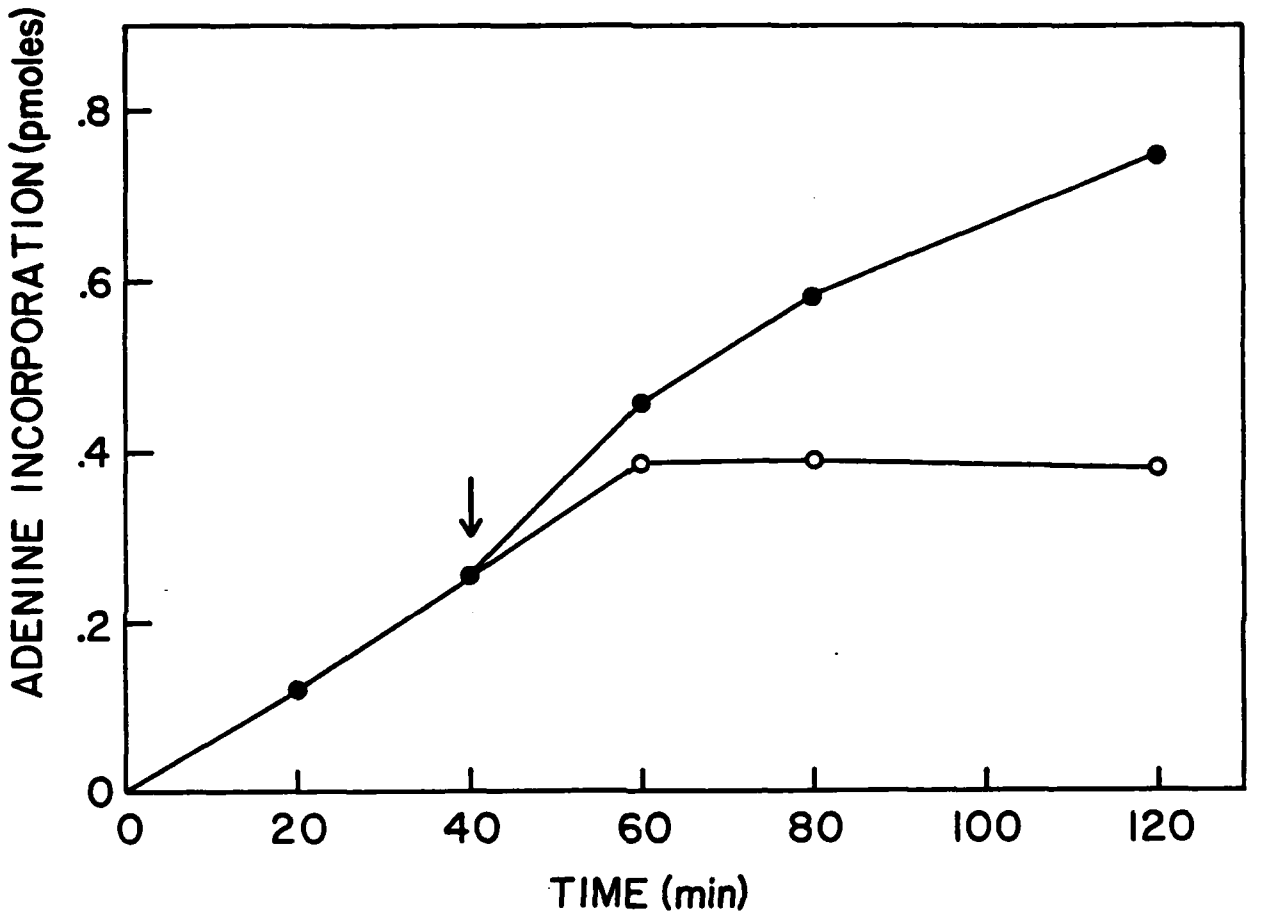


Figure 13. Adenine insertion into transfer RNA. [^{3}H]Adenine (1 μM) replaced [^{3}H]hypoxanthine in the standard reaction mixture with the transfer RNA-hypoxanthine ribosyltransferase from murine erythroleukemia (BB-88) cells. The time course of adenine incorporation into E. coli transfer RNA is depicted (●). Addition of 10 μM hypoxanthine at 40 minutes blocked further adenine incorporation (○).

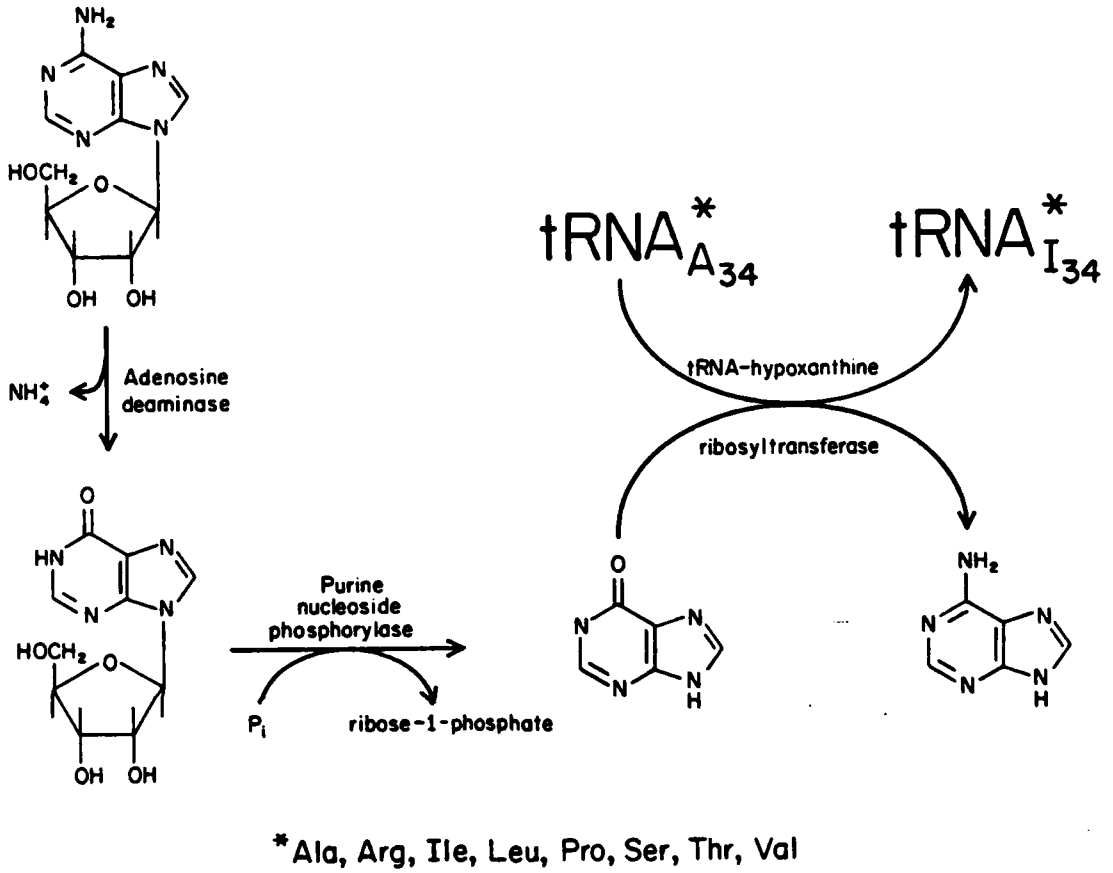


Figure 14. Inosine biosynthesis in the tRNA anticodon, and its relationship to purine catabolism. Adenosine is converted to inosine by adenosine deaminase, and inosine is converted to hypoxanthine by purine nucleoside phosphorylase. Hypoxanthine is exchanged for a base (probably adenine) in the first position of the anticodon (position 34 in Figure 1) by tRNA-hypoxanthine ribosyltransferase to generate inosine. Transfer RNAs for alanine, arginine, isoleucine, leucine, proline, serine, threonine, and valine are potential substrates in the reaction.

Table 1. Wobble hypothesis for base-pair interactions in tRNA and mRNA*

First anticodon nucleoside	Third codon nucleoside
C	G
A	U
U	A,G
G	U,C
I	U,C,A

*The purine and pyrimidine bases of the nucleosides listed in the table were predicted by Francis Crick to be able to base-pair in the tRNA anticodon and mRNA codon. The first anticodon nucleoside (i.e. the wobble position) is equivalent to position 34 in Figure 1. The nucleosides are: C, cytidine; A, adenosine; U, uridine; G, guanosine; and I, inosine.

cytidine with guanosine, and uridine with adenosine or guanosine (Table 1). Therefore, inosine in the anticodon expands the codon recognition potential of a particular tRNA.

Examination of the genetic code indicates which codons should permit inosine wobble with their tRNA counterparts (Figure 15). To insure fidelity of gene expression, only codons where uridine, cytidine, and adenosine in the third position all specify the same amino acid should be able to base pair with inosine-containing tRNAs. These codon families include those for the amino acids alanine, arginine, glycine, isoleucine, leucine, proline, serine, threonine, and valine, and with the exception of glycine, these are exactly the same amino acids accommodated by tRNAs known to contain inosine in the anticodon wobble position. Based on the total number of codons with the potential for interacting with inosine-containing tRNAs (Figure 15), our newly discovered tRNA modification reaction could have a major impact on gene expression.

There are also reasons to believe that the tRNA-hypoxanthine ribosyltransferase may be able to modulate gene expression. Hypoxanthine is known to induce differentiation of cultured murine erythroleukemia cells and human promyelocytic leukemia (HL-60) cells in vitro. Although hypoxanthine and certain purine analogs are effective inducers of erythroid differentiation, xanthine and uric acid are not. Therefore, specificity among the natural purine catabolites is indicated. The molecular basis for the hypoxanthine-induced differentiation has not been established, but it reportedly does not involve salvage into the cellular nucleotide pool by hypoxanthine-guanine phosphoribosyltransferase. Therefore, we have considered the possibility that hypoxanthine-induced differentiation may be mediated by tRNA-hypoxanthine ribosyltransferase. Until this enzyme was discovered (Elliott and Trewyn, 1984), the only known fate for hypoxanthine when added to cells was salvage into the cellular nucleotide pool or catabolism to xanthine and uric acid, and both of those possibilities were ruled out as being involved in the differentiation.

Major changes in tRNA isoaccepting species have been reported when cultured murine erythroleukemia cells are induced to differentiate with dimethylsulfoxide (DMSO). While some of these alterations in tRNA isoacceptors have been attributed to post-transcriptional changes in queuine modification, the structural basis for many of the changes has not been established. Included among these tRNAs are isoacceptors for the amino acids alanine, leucine, proline, serine, and threonine; tRNAs with the potential for having inosine in the first position of the anticodon. The tRNA isoaccepting species in the human HL-60 cells have not been examined during maturation induced by DMSO, hypoxanthine, or any other agent. However, the reported similarities between HL-60 cells and murine erythroleukemia cells with regard to induction of differentiation, suggest that the tRNA changes are likely to occur.

The similarity in the capabilities of DMSO and hypoxanthine to induce differentiation of immature blood cells in vitro has been somewhat confounding, since these compounds are so structurally dissimilar. However, they may elicit the identical structural change in the tRNA anticodon. DMSO is known to enhance the movement of materials across cell membranes, and hypoxanthine (like queuine) is a component of the sera utilized to supplement cell culture media in vitro. Therefore, DMSO may merely increase the intracellular availability of the substrate, hypoxanthine, to tRNA-hypoxanthine ribosyltransferase. Support for

First position	Second position				Third position
	U	C	A	G	
U	Phe	**Ser**	Tyr	Cys	U
	Phe	**Ser**	Tyr	Cys	C
	Leu	**Ser**	Stop	Stop	A
	Leu	**Ser**	Stop	Trp	G
C	**Leu**	**Pro**	His	**Arg**	U
	Leu	**Pro**	His	**Arg**	C
	Leu	**Pro**	Gln	**Arg**	A
	Leu	Pro	Gln	Arg	G
A	**Ile**	**Thr**	Asn	Ser	U
	Ile	**Thr**	Asn	Ser	C
	Ile	**Thr**	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	**Val**	**Ala**	Asp	**Gly**	U
	Val	**Ala**	Asp	**Gly**	C
	Val	**Ala**	Glu	**Gly**	A
	Val	Ala	Glu	Gly	G

Figure 15. The genetic code. Codons with the potential for interacting with tRNAs containing inosine in the wobble position are indicated by bold print for their amino acids. According to the wobble hypothesis (Table 1), inosine in the first position of the anticodon can base pair with uridine, cytidine, or adenosine in the third position of the codon, so only the codon families indicated would allow inosine wobble without altering the amino acid inserted during protein synthesis.

this possibility comes from the reported changes in queuine modification of tRNAs during DMSO-induced differentiation of murine erythroleukemia cells. A significant shift from queuine hypomodified to queuine modified tRNAs occurs early in the differentiation process, and it appears to be independent of changes in tRNA-guanine ribosyltransferase. Therefore, the enzyme is present in the undifferentiated cells, but the tRNA modification does not take place due to the lack of availability of queuine. Analogous compartmentalization may prevent inosine biosynthesis in the appropriate tRNA anticodons. We know that undifferentiated HL-60 cells contain significant levels of tRNA-hypoxanthine ribosyltransferase, since these cells were one of the sources for the enzyme in our original work (Elliott and Trewyn, 1984).

Growth curves for HL-60 cells treated with suboptional concentrations of DMSO and hypoxanthine alone and in combination are shown in Figure 16. Treatment with 100 mM DMSO alone diminished the growth of the HL-60 cells only slightly compared to the untreated controls, while 1 mM hypoxanthine was somewhat more effective. However, the combination of 100 mM DMSO plus 1 mM hypoxanthine resulted in an almost complete cessation of growth.

Although 1 mM hypoxanthine inhibited the growth of HL-60 cells (Figure 16), it was not effective at inducing differentiation (Table 2). Likewise, 100 mM DMSO alone caused no change in the proportion of cells exhibiting a more mature morphology when compared to the untreated controls. However, the combination of DMSO and hypoxanthine yielded a significant population of cells more mature than promyelocytes (Table 2). This synergistic induction of HL-60 differentiation is consistent with our proposal that DMSO enhances hypoxanthine access to the tRNA-hypoxanthine ribosyltransferase [Trewyn et al., submitted (appended)].

Enhanced protein synthesis (based on radiolabelled leucine incorporation) has been reported during the differentiation of HL-60 cells in vitro. In addition, the leucine radiolabelling of specific membrane-associated proteins was reported to be increased significantly during HL-60 differentiation into macrophage-like cells induced by phorbol esters. Therefore, we examined radiolabelled leucine incorporation by HL-60 cells treated with DMSO plus hypoxanthine to begin to examine the potential involvement of hypoxanthine insertion into $tRNA^{Leu}$ in the enhanced leucine labelling (Figure 17). As can be seen, the short-term exposure of HL-60 cells to DMSO plus hypoxanthine prior to adding radiolabelled leucine resulted in a significant increase in leucine incorporation into TCA-precipitable material. Neither DMSO alone nor DMSO plus adenine (a purine which should not effect an increase in tRNA inosine biosynthesis) caused similar increases (Figure 17). These results are also consistent with a potential role for the hypoxanthine insertion reaction (Trewyn et al., submitted).

The capability of hypoxanthine to induce differentiation of immature blood cells led to our evaluation of hypoxanthine as an inhibitor of the PDD-induced mimicry of transformation of normal human fibroblasts in vitro. As was demonstrated previously for queuine (Figure 8), hypoxanthine supplementation significantly inhibited the PDD-induced increase in saturation density (Figure 18). We have yet to compare the inosine content in the tRNAs from control and PDD treated fibroblasts, but based on the analogies to the queuine situation, PDD may well induce inosine (hypoxanthine) hypomodification as well.

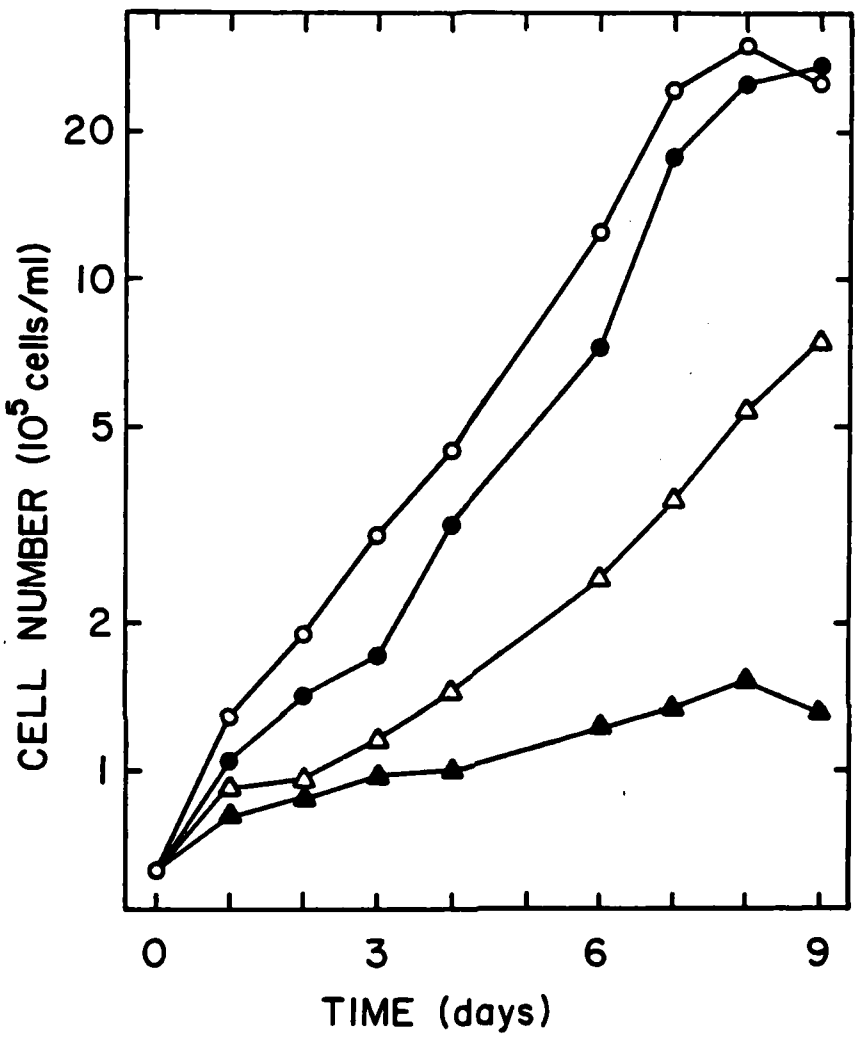


Figure 16. HL-60 growth curves. Cells plated at an initial density of 6×10^4 cells/ml were monitored for 9 days as described in Trewyn et al. [submitted (appended)]. The curves depict untreated control cells (○) as well as cells treated with 100 mM DMSO (●), 1 mM hypoxanthine (△), and 100 mM DMSO plus 1 mM hypoxanthine (▲).

Table 2. Differential counts of HL-60 cells after incubation with dimethylsulfoxide (DMSO) and/or hypoxanthine (Hx).*

Inducer	Percent of Total Cells			
	Promyelocytes	Myelocytes	Metamyelocytes	Neutrophils
None	89.4	7.8	2.8	0
DMSO	89.6	6.3	4.1	0
Hx	97.1	2.5	0.4	0
DMSO/Hx	46.2	32.4	19.5	1.9

* The cells were treated for 9 days with 100 mM DMSO, 1 mM Hx, or 100 mM DMSO plus 1 mM Hx. See Figure 17 and Trewyn et al. [submitted (appended)] for additional details.

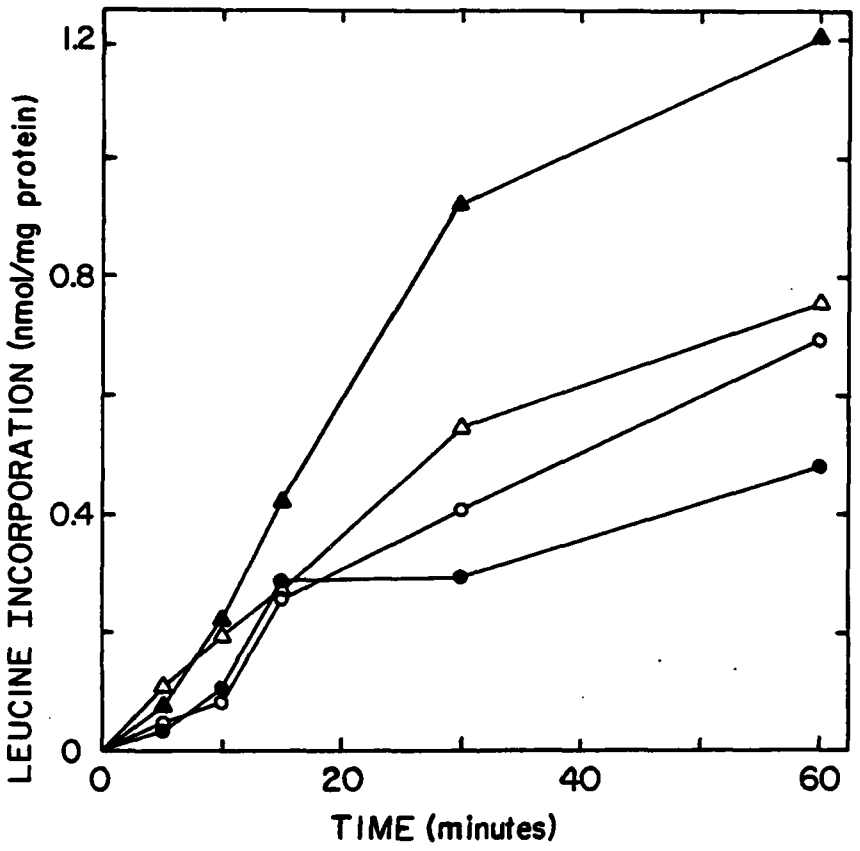


Figure 17. Enhanced leucine incorporation by HL-60 cells treated with DMSO plus hypoxanthine. Cells (5 x 10^5/ml) preincubated for 10 min in a Hank's balanced salt solution alone (O) or Hank's plus 210 mM DMSO (●), 210 mM DMSO and 1 mM adenine (Δ) or 210 mM DMSO and 1 mM hypoxanthine (▲) were radiolabelled with [^{14}C]leucine starting at time zero. At the time intervals indicated, samples were removed, and the amount of covalently incorporated leucine was determined by acid precipitation. See Trewyn et al. [submitted (appended)] for additional details.

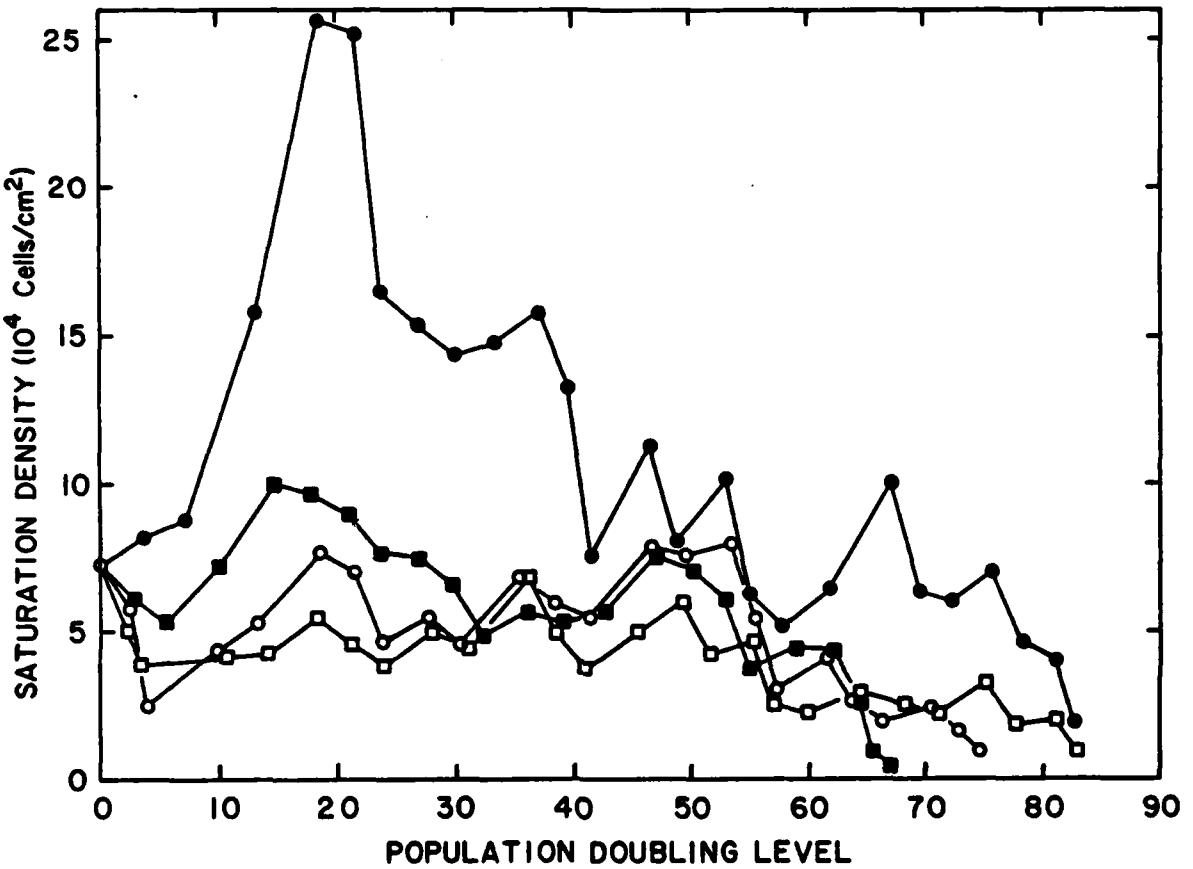


Figure 18. Saturation densities of human cells exposed continuously to PDD with or without excess hypoxanthine. The cell populations are: Control (0), control plus 3 x 10^{-3}M hypoxanthine (□), 10^{-8}M PDD (●), and 10^{-8}M PDD plus 3 x 10^{-3}M hypoxanthine (■).

A model depicting how inosine biosynthesis in tRNA might regulate protein synthesis is shown in Figure 19 (Trewyn et al., submitted). In the example illustrated, a leucine tRNA is modified to contain inosine, and that tRNA should be able to read the three leucine codons depicted in bold print (CUC,CUA, and CUU). The unmodified tRNA (with adenosine in the wobble position) should only read the last leucine codon (CUU), so the ribosome would stall (thereby blocking translation of the mRNA) if the anticodon modification were not carried out and leucine tRNAs capable of reading both of the other codons (CUC and CUA) were not present. Regulating protein synthesis by this means would be possible only because the genetic code is degenerate, so other essential mRNAs could use codons not read by inosine-containing tRNAs. For example, different mRNAs in the same cell (or cell compartment) depicted in Figure 19 could make use of the leucine codons CUG, UUA, and UUG, and as a result, their ability to function in protein synthesis would not be restricted. This model might help explain the effects of hypoxanthine on PDD-induced mimicry of transformation with human fibroblasts (Figure 18) as well as the effects of DMSO plus hypoxanthine on HL-60 cells (Figures 16 and 17).

In summary, it was proposed in 1980 that changes in tRNA metabolism are required for cells to progress through the stages of carcinogenesis, and significant progress was made towards demonstrating such a requirement. A comprehensive hypothesis was formulated to describe tRNA-mediated endogenous promotion of carcinogenesis, and this hypothesis offers an explanation for the lengthy time frame observed between carcinogen exposure and neoplastic transformation. A role was defined for 7-methylguanine as an endogenous promoting agent, whereby this natural RNA catabolite induces queuine hypomodification in the tRNA anticodon by inhibiting the queuine insertion enzyme tRNA-guanine ribosyltransferase. Subsequently, 7-methylguanine induces neoplastic transformation.

A cell culture system was developed which allows (for the first time) the study of tumor promoter-induced mimicry of transformation with normal human cells, and using this system, phorbol ester tumor promoters were also demonstrated to induce queuine hypomodification of tRNA. However, in this case, the hypomodification occurred due to a specific phorbol ester inhibition of queuine transport into the cells. Most importantly, overcoming the tumor promoter-induced hypomodification of tRNA by supplying the cells with excess queuine, blocked the expression of a transformed phenotype by the human cells. Therefore, queuine may be an anti-promoting compound, and a role for queuine hypomodification in the expression (promotion) of carcinogenesis appears likely.

Finally, a "new" tRNA modification enzyme was discovered; an enzyme involved in the biosynthesis of inosine in the first (wobble) position of the anticodon of selected tRNAs. This hypoxanthine insertion enzyme, a tRNA-hypoxanthine ribosyltransferase, may have an important role in regulating protein synthesis, since inosine in the wobble position greatly expands the mRNA codon recognition potential of that tRNA. Evidence was obtained that the hypoxanthine insertion reaction may be involved in cell differentiation, and that defects in insertion may be involved in the dedifferentiation associated with neoplasia. Again, the results are consistent with our model for tRNA-mediated promotion of carcinogenesis.

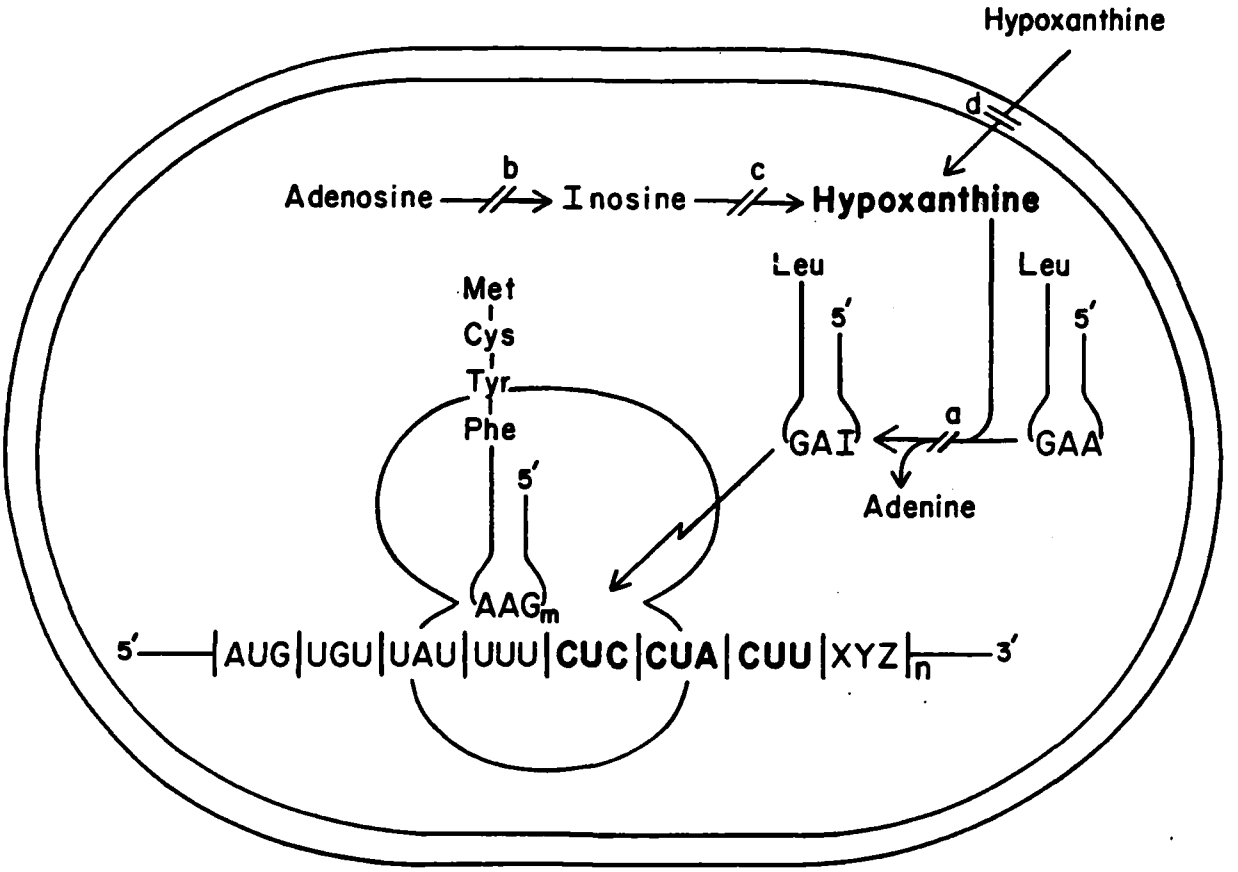


Figure 19. Postulated model for how the hypoxanthine insertion reaction in specific tRNA anticodons may regulate protein synthesis. Transfer RNAs with the potential for having hypoxanthine inserted into the first position of the anticodon include those for alanine, arginine, isoleucine, leucine, proline, serine, threonine, valine, and perhaps glycine. Any of these tRNA species with adenine in the primary transcript wobble position could be involved in regulating translation as shown. However, in the example illustrated, a leucine tRNA is modified by tRNA-hypoxanthine ribosyltransferase (a) which, according to the wobble hypothesis (Table 1), should allow the resulting inosine-containing tRNAs to read the three leucine codons depicted in bold print (CUC, CUA, and CUU). The unmodified tRNAs should only read the last leucine codon (CUU), so the ribosome would stall if the anticodon modification were not carried out. The substrate for inosine biosynthesis in tRNA, hypoxanthine, could be generated endogenously from adenosine and inosine by the enzymes adenosine deaminase (b) and purine nucleoside phosphorylase (c), or it could be supplied exogenously and transported (d) into the cell or cell compartment.

D. Publications (appended)

1. Elliott, M.S. and Trewyn, R.W. Queuine hypomodification of tRNA induced by 7-methylguanine. Biochem. Biophys. Res. Commun. 104: 326-332, 1982.

2. Milo, G.E. and Trewyn, R.W. In vitro transformation of cultured human diploid fibroblasts. In: Nitrosamines and Human Cancer (P.N. Magee, ed.), Banbury Report 12, pp. 3-13, Cold Spring Harbor Laboratory, 1982.

3. Trewyn, R.W., Elliott, M.S., Glaser, R., and Grever, M.R. Alterations in tRNA metabolism as markers of neoplastic transformation. In: Biochemical and Biological Markers of Neoplastic Transformation (P. Chandra, ed.), pp. 263-276, Plenum Publishing Corp., New York, 1983.

4. Davakis, L.A. and Trewyn, R.W. Evaluating tumor promoter activity in vitro with human diploid fibroblasts. In: Polynuclear Aromatic Hydrocarbons: Formation, Metabolism, and Measurement (M. Cooke and A.J. Dennis, eds.), pp. 393-404, Battelle Press, Columbus, 1983.

5. Elliott, M.S. and Trewyn, R.W. Inosine biosynthesis in transfer RNA by an enzymatic insertion of hypoxanthine. J. Biol. Chem. 259: 2407-2410, 1984.

6. Trewyn, R.W. and Gatz, H.B. Altered growth properties of normal human cells induced by phorbol 12,13-didecanoate. In Vitro 20: 409-415, 1984.

7. Elliott, M.S., Katze, J.R., and Trewyn, R.W. Relationship between a tumor promoter-induced decrease in queuine modification of transfer RNA in normal human cells and the expression of an altered cell phenotype. Cancer Res. 44: 3215-3219, 1984.

8. Elliott, M.S., Trewyn, R.W., and Katze, J.R. Inhibition of queuine uptake in cultured human fibroblasts by phorbol 12,13-didecanoate. Cancer Res. (in press).

9. Trewyn, R.W., Kretz, K.A., Utz, E.D., Patrick, D.E., and Muralidhar, G. Hematopoiesis and the inosine modification in transfer RNA. Proc. Soc. Exp. Biol. Med. (submitted).

E. Personnel

Ronald W. Trewyn, Ph.D., Associate Professor of Physiological Chemistry, Principal Investigator.

Holly B. Gatz, Technician (11/80 to 8/82)

Eric D. Utz, Technician (8/82 to 9/84)

Dawn E. Gibboney, Technician (10/83 to 9/84)

Lani A. Davakis, M.S., 1983
Thesis: Response of Human Cells to Tumor Promoters

Mark S. Elliott, Ph.D., 1983
Thesis: Biosynthesis of Queuosine and Inosine in the First Position of the Anticodon in Transfer RNA: Implications in Neoplasia.

Keith A. Kretz, Ph.D. Candidate

F. Interactions

NATO International Advanced Study Institute, Corfu Island, Greece, September 28, 1981 to October 10, 1981.

Presentation: Alterations in tRNA Metabolism as Markers of Neoplastic Transformation.

Publication: See Section D, Number 3.

American Association for Cancer Research, St. Louis, Missouri, April 28, 1982 to May 1, 1982.

Presentation: Putative Tumor Promoter Response in Cultured Human Diploid Cells.

Published Abstract: Trewyn, R.W., Gatz, H.B., Davakis, L.A., and Elliott, M.S. Proc. Amer. Assoc. Cancer Res. 23: 103, 1982.

Seventh International Symposium on Polynuclear Aromatic Hydrocarbons, Battelle's Columbus Laboratories, Columbus, Ohio, October 26-28, 1982.

Presentation: Evaluating Tumor Promoter Activity in Vitro with Human Diploid Fibroblasts.

Publication: See Section D, Number 4.

American Society of Biological Chemists, San Francisco, California, June 5-9, 1983.

Presentation: Enzymatic Insertion of Hypoxanthine into Transfer RNA.

Published Abstract: Elliott, M.S. and Trewyn, R.W. Fed. Proc. 42: 2149, 1983.

Review of Air Force Sponsored Basic Research in Biomedical Sciences, Irvine, California, July 26-28, 1983.

Presentation: Transfer RNA-Mediated Endogenous Promotion of Carcinogenesis.

American Association for Cancer Research, Toronto, Canada, May 9-12, 1984.

Presentation: Inosine Biosynthesis in Transfer RNA: A Postulated Defect in Immunodeficiency Diseases and Leukemia.

Published Abstract: Kretz, K.A., Elliott, M.S., Utz, E.D., and Trewyn, R.W. Proc. Amer. Assoc. Cancer Res. 25: 23, 1984.

American Society of Biological Chemists, St. Louis, Missouri, June 3-7, 1984.

Presentation: Phorbol Ester Inhibition of Quenine Uptake by Human Fibroblasts in Culture.

Published Abstract: Elliott, M.S., Trewyn, R.W. and Katze, J.R. Fed. Proc. 43: 1780, 1984.

G. Appendix

Publications listed in Section D.

Vol. 104, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1982 Pages 326-332

QUEUINE HYPOMODIFICATION OF tRNA INDUCED BY 7-METHYLGUANINE

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Comprehensive Cancer Center
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Received December 1, 1981

SUMMARY: Transfer RNA isolated from Chinese hamster cells transformed by 7-methylguanine is hypomodified for queuine. 7-Methylguanine rapidly induces queuine hypomodification of tRNA in normal Chinese hamster embryo cells under conditions leading to transformation, and the enzyme catalyzing the queuine modification reaction, tRNA: guanine transglycosylase, is inhibited by 7-methylguanine *in vitro*.

Extensive post-transcriptional modification of tRNA generates macromolecules containing a vast array of altered purines and pyrimidines. Queuine (Q-base), a highly modified guanine analog, is one such modification that is found in the first position of the anticodon of tRNA isoacceptors for histidine, tyrosine, aspartic acid, and asparagine (1). Transfer RNA isolated from normal mammalian cells is almost fully modified for Q-base. However, tRNA from tumor tissue or cells transformed *in vitro* exhibits pronounced hypomodification for Q-base (2). This structural change in the anticodon leads to the appearance in transformed cells of unique tRNA isoaccepting species with altered chromatographic characteristics *in vitro* (2-4).

The enzymatic modification generating Q-containing tRNA is unusual, in that it involves a direct replacement of Q-base for guanine (5,6). The enzyme responsible for catalyzing this reaction is tRNA: guanine transglycosylase (5,6), and a generalized reaction scheme for the mammalian enzyme is depicted in Figure 1. The enzyme was discovered in rabbit reticulocytes by its ability

Abbreviations: Q-base, queuine [7-(3,4-trans-4,5-cis-dihydroxy-1-cyclopenten-3-ylamino- methyl)-7-deazaguanine]; ChH, normal Chinese hamster embryo cells; ChH-1G, Chinese hamster embryo cells transformed with 1-methylguanine. ChH-7G, Chinese hamster embryo cells transformed with 7-methylguanine.

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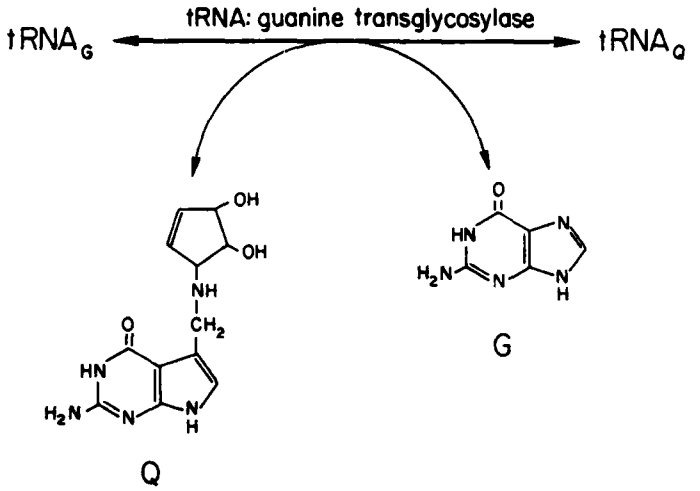

Fig. 1. tRNA: guanine transglycosylase reaction responsible for exchanging Q-base for guanine in the first position of the anticodon of mammalian tRNA for asparagine, aspartic acid, histidine and tyrosine. The abbreviations are: Q, 7-(3,4-trans-4,5-cis-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanine (queuine); and G, guanine.

to insert radiolabeled guanine into homologous tRNA (7), and with appropriate Q-containing tRNA substrates, the reaction is reversible in vitro (8).

Bacterial tRNA also contains Q-base. However, the tRNA: guanine transglycosylase from Escherichia coli utilizes a precursor of Q-base, 7-(aminomethyl)-7-deazaguanine, as the initial substrate, and then the modification is completed at the macromolecular level (9). E. coli tRNA: guanine transglycosylase has been characterized extensively (2,9,10), and it has been shown to be inhibited very effectively by 7-methylguanine (10).

It was demonstrated previously that chronic exposure of normal Chinese hamster embryo cells to 7-methylguanine or 1-methylguanine can lead to altered cellular growth properties, including in vitro neoplastic transformation (11-13). How normal components of cellular nucleic acids (7-methylguanine and 1-methylguanine) can influence phenotypic expression when supplied to the cell's culture environment has not been established. However, the reported inhibition of E. coli tRNA: guanine transglycosylase by 7-methylguanine led us to investigate whether the methylated purines transforming Chinese hamster cells could inhibit Q-base modification of tRNA. It was surmised that a major structural change (guanine vs Q-base) in the anticodon of specific tRNA species

might play some role in altered gene expression in cells exposed to elevated methylated purine concentrations for prolonged periods.

MATERIALS AND METHODS

Establishment and propagation of primary cultures of Chinese hamster embryo (ChH) cells were as previously described except that the culture medium was supplemented with 5% fetal bovine serum (11,12). Establishment of transformed ChH cell lines by chronic exposure to 10 μM 1-methylguanine (ChH-1G) and 10 μM 7-methylguanine (ChH-7G) was reported previously (11). Fetal bovine serum deficient in Q-base was prepared by treatment with dextran-coated charcoal as described by Katze (3). Proliferating cells were homogenized in hypotonic buffer, and tRNA was isolated as described by Wilkinson and Kerr (14).

Transfer RNA: guanine transglycosylase was isolated from E. coli MRE 600 cells (Grain Processing Corp.) as described by Okada and Nishimura (10). However, purification of the enzyme was carried only through the DEAE-cellulose chromatography step, since the preparation was free of RNase activity when the RNase-deficient strain MRE 600 was utilized. The E. coli tRNA: guanine transglycosylase was used to assay for Q-hypomodified tRNA as reported by Okada et al. (2). The assay procedure was that of Howes and Farkas (15) with a modified reaction mixture containing: 10 μmoles Tris-HCl (pH 7.4), 53 μmoles KCl, 5 μmoles 2-mercaptoethanol, 1 nmole [8-^{3}H]guanine (1 Ci/mmole), 0.05 to 0.25 A_{260} units tRNA, and 6 units E. coli tRNA: guanine transglycosylase in a total volume of 0.6 ml. Transfer RNA was precipitated and collected on glass fiber filters for scintillation counting (15).

Rabbit erythrocyte lysates were prepared as the source of a mammalian tRNA: guanine transglycosylase as described by Howes and Farkas (15). The lysate was centrifuged at 20,000 x g for 20 minutes and 105,000 x g for 90 minutes and desalted on a Sephadex G-25 column. This enzyme preparation was used to assay 1-methylguanine and 7-methylguanine as enzyme inhibitors. The reaction mixture was as described above for Q-hypomodified tRNA using the E. coli enzyme except 2.0 A_{260} units of Q-deficient yeast tRNA was included as a substrate and the guanine concentration was 1 μM. In this case, the reaction was terminated and extracted with phenol as described by Farkas and Singh (7) before precipitating and collecting the tRNA on glass fiber filters.

RESULTS AND DISCUSSION

Transfer RNA isolated from transformed ChH-1G and ChH-7G cells was assayed for deficiency of Q-base using the E. coli enzyme, and the results are presented in Table 1. The tRNAs from cells transformed by the methylated purines were approximately 5-fold better tRNA: guanine transglycosylase substrates than the tRNA from normal ChH cells. The tRNAs from the transformed cells were also better substrates in the assay than the positive control, yeast tRNA.

To determine whether 1-methylguanine and/or 7-methylguanine can inhibit Q-base modification of cellular tRNA directly, normal ChH cells were exposed to the individual methylated purines for six population doublings. E. coli tRNA:

TABLE I. Queuine-hypomodified tRNA from Chinese hamster cells transformed by methylated purines.

tRNA Source	Guanine Incorporation (pmoles/hr/A_{260} unit)[a]
ChH	3.9 ± 0.4
ChH-1G	17.6 ± 1.5[b]
ChH-7G	19.9 ± 6.8[b]
Yeast	6.6 ± 1.8
E. coli	<0.2

[a] Mean ± standard deviation from 4 experiments.

[b] Significantly different from normal ChH cells; $P \leq 0.05$.

guanine transglycosylase was again used to assay for deficiency of Q-base in the tRNA isolated from the cells, and these results are presented in Figure 2. As can be seen, the short-term exposure of the normal diploid cells to 10 μM 7-methylguanine led to the induction of Q-deficient tRNA. However exposure to

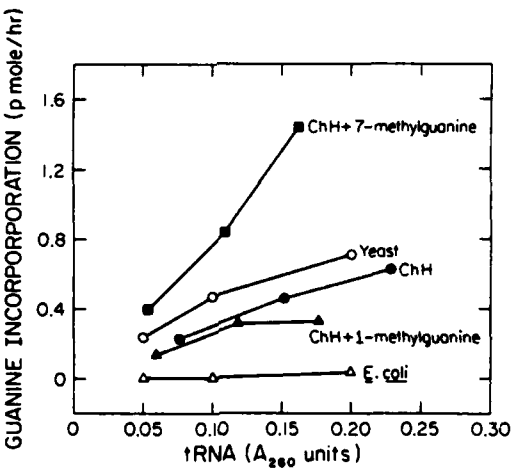

Fig. 2. Hypomodification of ChH tRNA for Q-base induced by 7-methylguanine. Transfer RNA was isolated from ChH cells treated for 6 population doublings with 10 μM 7-methylguanine (■), from ChH cells treated for 6 population doublings with 10 μM 1-methylguanine (▲), and from matched untreated ChH cells (●). Yeast tRNA (○) and E. coli tRNA (△) were used as Q-deficient and Q-sufficient controls respectively. Triplicate reactions were run at each tRNA concentration. See Materials and Methods for additional details.

10 μM 1-methylguanine did not lead to Q-hypomodification of tRNA after six population doublings (Figure 2).

It has been reported that mouse cells either cannot synthesize Q-base or cannot synthesize enough for their needs (16), and therefore, cells in culture obtain most of this modified purine from the serum utilized to supplement the growth medium (5,17). To establish whether ChH cells also require exogenous Q-base for proper tRNA maturation, cells were grown for two population doublings in Q-deficient serum after which the tRNA was assayed for Q-hypomodification. The results are presented in Figure 3, and the requirement for exogenous Q-base is clearly evident.

Transfer RNA: guanine transglycosylase from rabbit erythrocytes was used to assess the direct inhibition of tRNA modification in vitro by 1-methylguanine and 7-methylguanine. These results are presented in Figure 4. As can be seen, 10 μM 7-methylguanine effectively inhibited the mammalian enzyme, whereas 1-methylguanine was much less effective. These results for the isolated rabbit erythrocyte enzyme are consistent with those for the cultured ChH cells presented in Figure 2. However, it remains to be established whether the induction of Q-hypomodification of ChH tRNA by 7-methylguanine occurs by a direct inhibition of the ChH transglycosylase or by the inhibition of transport of exogenous Q-base. These studies are underway, as are examinations of the kinetics of enzyme inhibition.

The possibility that tRNA: guanine transglycosylase inhibitors are responsible for the Q-hypomodification of tRNA in malignant cells in vivo was suggested by other investigators based on comparable enzyme activities being observed in normal Q-sufficient and malignant Q-deficient tissues (6,18). The nature of these putative inhibitors was not established. However, it is known that malignant tissues contain aberrant tRNA methyltransferases (19), and that cancer patients excrete highly elevated levels of tRNA catabolites; especially methylated derivatives (19,20). Therefore, it is possible that increased endogenous methylated purines (e.g. 7-methylguanine) may be involved in inducing the Q-hypomodification of tRNA associated with neoplasia. This could

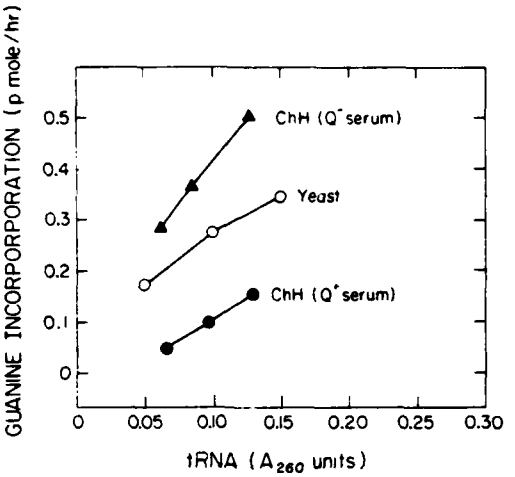

Fig. 3. Hypomodification of ChH tRNA for Q-base induced by culturing cells in Q-deficient serum. Transfer RNA was isolated from ChH cells grown for 2 population doubling in Q-deficient (Q^-) serum (▲) or matched Q-sufficient (Q^+) serum (●). See legend to Figure 2 and Materials and Methods for additional details.

explain how Q-deficient tRNA is generated in normal tissues far from the tumor origin in animals (21). In addition, it offers an explanation for the source of Q-deficient tRNA in the ChH-1G cells, since it was demonstrated previously that the tRNA methyltransferase activity in these cells is elevated significantly (11).

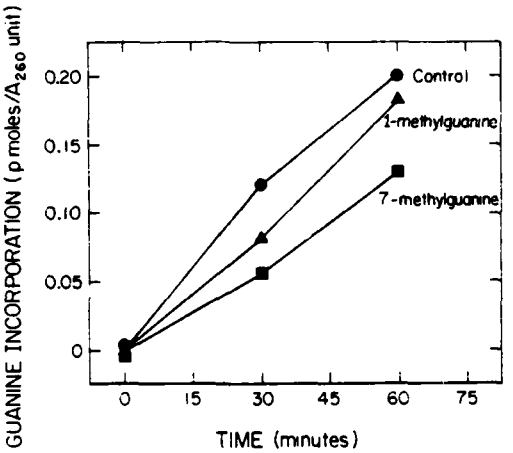

Fig. 4. Inhibition of tRNA: guanine transglycosylase from rabbit erythrocytes by methylated guanines. The assays were carried out as described in the Materials and Methods with the guanine substrate concentration at 1 μM. The methylguanines were added at time zero at a final concentration of 10 μM. The reactions are: Control (●), plus 1-methylguanine (▲), and plus 7-methylguanine (■).

Studies are underway to determine if inhibition of tRNA modification by 7-methylguanine or other methylated purines excreted at high levels by cancer patients has some fundamental role in the expression of carcinogenesis. A model invoking such a role was proposed previously (22).

ACKNOWLEDGMENTS

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Reprinted from *Banbury Report 12: Nitrosamines and Human Cancer*

In Vitro Transformation of Cultured Human Diploid Fibroblasts

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The carcinogenicity of nitrosamines and nitrosamides was reported to be dependent upon the alkylation of macromolecules following the breakdown of the nitroso compounds (Loveless 1969). In mammalian rodent systems, a carcinogenic event occurred following methylation of the DNA (Magee and Hultin 1962; Magee and Farber 1962). Loveless (1969) suggested that the O^6 position of DNA-guanine is quantitatively alkylated by *N*-nitroso compounds. Gerchman and Ludlum (1973) suggested that methylated-O^6 guanine is read not as a guanine but adenine, therefore a misspelling occurs at a critical site in the DNA with a subsequent carcinogenic response. Subsequently, Goth and Rajewsky (1974) indicated that the persistence of this error was the most important step in the carcinogenesis process and that the alkylation must persist until the DNA replicates semiconservatively and the daughter cells receive this aberrant DNA.

In human cells treated with a carcinogen (Milo et al. 1978a,b) we recognized that damage to DNA by these agents was repaired quickly by error-free repair systems (Milo and Hart 1976). Over the years attempts to reproducibly transform randomly proliferating human diploid cell populations has met with failure. However, we describe a program for the induction of carcinogenesis in vitro with human diploid fibroblasts using nitroso compounds as the instruments to deliver a carcinogenic insult. The biological endpoint measuring the carcinogenic insult will be an expression of anchorage-independent growth, cellular invasiveness and neoplasia in a xenogeneic host.

METHODS

Chemicals

The chemicals of interest for this study are dimethylnitrosamine, (NDMA, $CH_3N(NO)CH_3$); diethylnitrosamine (NDEA, $CH_3CH_2N(NO)CH_3$); *N*-methyl-*N'*-Nitro-*N*-nitrosoquanidine (MNNG); ethylnitrosourea (ENU, $C_2H_5N(NO)$-$CONH_2$); and methylnitrosourea (MNU, $CH_3N(NO)CONH_2$). These chemicals

were furnished by the NCI Chemical Repository DCCP-NCI for this study sponsored by the National Cancer Institute. The modulators were obtained from commercial sources: insulin (Sigma Chemical, St. Louis, MO), phorbol myristate acetate, (Consolidated Midland Corp., Brewster, NY) and anthralin, (Sigma Chemical Co., St. Louis, MO).

Cell Cultures

Neonatal foreskin fibroblast cell populations were prepared as described previously, (Riegner et al. 1976; Oldham et al. 1980). Briefly, the fibroblasts were separated immediately from the mixed culture by selective detachment from the epithelial cells attached to a plastic substratum. The fibroblast cultures were passaged routinely using 0.1% trypsin and the subsequent cultures were maintained on Eagles-minimum essential medium (MEM) prepared with Hanks' balanced salt solution, 25.0mM HEPES buffer (GIBCO, Grand Island, NY) at pH 7.2. Additional supplements were added as needed (Riegner et al. 1976). The cultures were incubated in an atmosphere of 4% CO_2-enriched air atmosphere at 37°C.

Transformation Procedure

Toxicity Protocol

The transformation procedure is broken down into two distinct operational procedures: the cytotoxicity evaluation of each suspect carcinogen and the transformation protocol. Each chemical regardless of its structural similarity must be evaluated for its cytotoxic effects. Foreskin fibroblasts were isolated from the tissue (Oldham et al. 1980; Allred et al. 1982) and seeded at the first passage at 40 cells/cm^2 in MEM supplemented with growth additives, (Milo and DiPaolo 1978) Complete Media V (CMV), and incubated at 37° in a 4% carbon dioxide-enriched air environment and 10% FBS. The chemicals of interest are added to the populations for a 16-hour period at different concentrations of the chemical. The experimental medium was removed and replaced with MEM supplemented with 20% FBS, (Milo and DiPaolo 1980; Oldham et al. 1980). Two weeks later the cultures were fixed in 10% phosphate-buffered formalin and were stained with hematoxylin and eosin, and enumerated manually or on a differential image optical analyzer (Gavino et al. 1982). The data is expressed as relative colony forming efficiency i.e., the number of colonies 10 cells in size or larger that formed within 21 days of concluding treatment divided by the number of colonies that formed in the untreated populations X 100.

Transformation Protocol

The concentrations of the compound of interest found to give an effective cytotoxic dose of 50%, 25% or noncytotoxic dose on cells at 40 cells/cm^2 were used

as the concentrations of choice to initiate the transformation process. We run every treatment in triplicate (i.e., on 3 different cultures derived from an initial culture prior to population doubling [PDL] 6). The randomly logarithmically growing cultures at 5000 cells/cm^2 are seeded into a Dulbecco's Modified MEM minus arginine and glutamine and supplemented with dialyzed-FBS for 16 hours. The 2-hour-initiated thymidine radiolabeling index decreases from 18-23% to 0.1% to 0% in that time, (Milo and DiPaolo 1980; Oldham et al. 1980). At that time the cells are released from the G_1 block by the readdition of CMV-10% FBS containing 2mM glutamine and 1mM arginine plus either modulator 10U/ml insulin 0.1μg/ml anthralin or 5×10^{-7}M PMA. Ten hours following release from the G_1 block the cells exhibit S phase entry. The carcinogens of interest were added and left on for 12 hours. At the conclusion of the treatment the experimental medium was removed and replaced with CMV supplemented with 10% FBS. The cells were allowed to recover for 2 days and then were split 1:2 into CMV containing 2x vitamins, 8x nonessential amino acids and 20% FBS, hereafter referred to as 8x medium. When these treated cultures reached 80% to 90% confluent density they were serially passaged 1:10 into 8x medium until PDL-20.

Characterization of Transformed Cells:

Anchorage Independent Growth

The carcinogen populations at PDL 20 were seeded at 50,000 cells in 2ml of 0.33% agar, (in a 25cm^2 well) prepared in Dulbecco's LoCal medium (Biolabs, Northbrook, IL) supplemented with additives, (Milo et al. 1981c) and 20% FBS. This cell suspension was layered over 5ml of a 2% agar base prepared in RPMI 1629 medium (GIBCO) plus additives, (Milo et al. 1981a,c). The seeded cultures were not disturbed for 1 week and were subsequently observed on a weekly basis for 3-4 weeks. Cultures were scored as positive when colonies of > 50 cells were observed. Colonies were removed and reestablished in culture. After attachment and growth to ~20% density the cells were trypsinized and reseeded to distribute evenly over the substratum.

Cellular Invasiveness

The carcinogen-treated population that formed colonies in soft agar were reestablished in culture and subsequently evaluated for tumor potential, (Noguchi et al. 1978; Milo et al. 1981c), using chick embryonic skin (CES) in vitro. The CES organ culture was modified to optimize sensitivity to the transformed cells and frequency of success for a rapid assay for cellular neoplasia. Eggs were incubated for 9-10 days in a humidifed egg incubator. The embryos were removed from the eggs, the skins separated from the dorsal part of the embryo and placed on an agar base containing 10 parts of 1% agar in Earle's balanced salt solution, 4 parts FBS, and 4 parts chick embryo extract. The treated cells,

250,000 in number contained in 0.04μl of MEM, were added to the CES organ culture. These cultures were incubated in a humidified incubator at 37°C in a 4% CO_2 enriched air atmosphere. On day 4 the skins were removed and fixed in Bouin's solution. The stained 5μM sections on slides were examined by light microscopy (Milo et al. 1981a).

Tumor Growth in Nude Mice

Treated populations that exhibited anchorage independent growth were evaluated in 6 week old nude mice (Sprague-Dawley). The mice were irradiated with 137CS source at 450 RAD whole body irradiation 48 hours prior to subcutaneous injection of 5×10^6 cells. Six weeks later the tumors were counted and the incidence of tumor formation recorded (Milo et al. 1981a).

RESULTS

The cell population treated with the individual compounds included herein were treated at equivalent cytotoxic doses to transform the human foreskin fibroblasts. It has been found that treatment regimens for transformation protocols were more reliable when the populations at risk to the suspect carcinogenic agent were treated at $ED_{10,25 \text{ or } 50}$ toxic doses or noncytotoxic equivalent doses rather than chemical equivalent doses. In all experiments where toxic values exceeded the ED_{50} values the incidence of transformation as measured by anchorage independent growth and cellular invasiveness tended to drop to zero (control values). Once the toxicity of these compounds has been evaluated then the toxicity of the modulators has to be determined. All modulators were used at ED_0 doses, i.e. a noncytotoxic dose. The problem one was faced with in these comparisons were the integrated effects that the modulators have on cell permeability. This was why we used these modulators on the cells at exceedingly low concentrations to elicit the proper response without interfering with the cytotoxic dose of the carcinogens of interest (Table 1).

Table 1
Cytotoxicity of Nitrosamine Derivatives Determined on Human Foreskin Fibroblasts In Vitro

Chemical Compound	Cytotoxic Effect ED 50 in 4 (μg/ml)
NDMA	0.001
NDEA	0.01
MNU	29.0
ENU	44.0
MNNG	0.1

These concentrations represent the cytotoxic dose that yielded a 50% inhibition of the relative cloning efficiency. (Data from Allred et al. 1982).

Early Stage

Treated populations responded to the carcinogenic insult in a different predictable manner than populations just responding to toxic insults. First, for example, populations responding to toxic levels of modulators while exhibiting selective changes in plasma membrane permeability, did not exhibit altered lectin agglutination profiles as seen by populations responding to a carcinogenic insult.

Once carcinogen-treated populations exhibited these features they were serially passaged for PDL 20 before seeding in soft agar.

Transitional Stage

Using the growth medium we described under transformation protocol (Material and Methods section), the optimum time for seeding the treated populations in soft agar was 20 PDL following treatment with the carcinogen. Second, it was interesting to note that at this PDL; NDMA-, NDEA-, and MNNG-treated populations exhibited anchorage independent growth while ENU- and MNU-treated populations did not exhibit this feature. NDMA induced colony formation in soft agar of 13 colonies/10^5 cells; NDEA, 8 colonies/10^5 cells and MNNG, 1 colony/10^5 cells. The colonies 50 cells in size or larger were removed and reseeded in flasks. At a 80% preconfluent density the cells were seeded onto CES and 4 days later evaluated (Table 2). The treated populations that exhibited anchorage independent growth also exhibited cellular invasiveness.

Table 2
Evaluation of Different Nitrosamine-Treated Cell Populations for Anchorage-Independent Growth, Cellular Invasiveness, and Tumor Incidence

Chemical compound	Cytotoxic effect (ED 50 μg/ml)[a]	Cellular invasiveness[b]	Anchorage-independent growth[c]	Tumor incidence[d]
NDMA	0.001	+	13	2/6
NDEA	0.01	+	8	3/8
MNU	29.0	-	0	N.D.
ENU	44.0	-	0	N.D.
MNNG	0.1	+	1	3/5

[a]Represents the cytotoxic dose that yielded a 50% inhibition of the relative cloning efficiency.

[b]A positive response indicates that out of 6 CES organ cultures one or more sections upon examination by the pathologist exhibited cellular invasiveness.

[c]Colonies 50 cells or more in size were scored as positive when counted 21 days post-seeding in the soft agar overlayer. Each 25 cm^2 well was seeded with 50,000 cells, (Methods section).

[d]The numerator represents the number of positive takes (tumors) over the number of mice injected with 5×10^6 treated cells. (Methods section).

Late Stage

At this time the different treated populations were injected in nude mice and the incidence of tumor formation noted. Injected NDMA-treated cells elicited an incidence of tumor formation in 2 out of 6 mice; NDEA-treated cells 3 out of 8 mice and MNNG treated cells 3 out of 5 mice. The tumors were examined by histopathology and interpreted to be undifferentiated mesenchymal tumors. The cellular tumors evaluated from the CES were described as simulated firbosarcomas. This definition was used because the pathologist was describing cellular invasion into CES, an organ culture, in vitro. The two interpretations are not incompatible.

DISCUSSION

The data here were presented to illustrate the sequencing that can optimize the program of human foreskin fibroblast response to a carcinogenic insult in vitro. These compounds that were evaluated for their carcinogenic potential appear to require cell proliferation for the fixation of the carcinogen damage. Once the insult is fixed, a program of selective expression of the initiated cells occurs over a protracted time period, PDL 20, followed by anchorage independent growth of the transformed cells.

A similar selection pressure is also expressed by the cell population prior to carcinogen treatment, i.e. cells passaged in culture > 10 PDL are refractory to a carcinogenic insult (Sutherland, et al. 1980; Milo et al. 1981c; Zimmerman and Little et al. 1981). These observations are not unusual in themselves, for example, we have seen the same refractoriness exhibited by human foreskin populations to feline sarcoma virus-directed transformations in vitro (Milo et al. 1981b) at high PDL. These vector-directed transformations also exhibit multistage (early, transitional, and late stage of expression of carcinogenesis) carcinogenesis exhibited by the nitrosamine-transformed cell populations. An interesting feature of the chemical carcinogen-induced transformation of human cells as seen by Silinskas et al. (1981) was that they used a different medium to culture their cells in soft agar and observe colony formation around 8-13 PDL following carcinogen treatment rather than PDL 20. Zimmerman and Little (1981), and Sutherland et al. (1980) observed also that cells treated at PDL > 10 were refractory to the carcinogenic insult. Recently, Tejwani et al. (1982) demonstrated that DNA-adducts formed by benzo[*a*] pyrene (B[*a*]P) or B[*a*]P 9,10-ene diol epoxide anti form of B[*a*]P in susceptible populations were qualitatively similar to the principal adduct formed in the refractory cells i.e. B[*a*]P 9,10-ene diol epoxide-deoxyguanosine adduct.

It is our contention that optimum sensitivity occurs in S-phase to the carcinogen insult and that the response can be amplified by the addition of modulators (Milo and DiPaolo 1980). These events are followed by the persistence of the adducts during the critical point of the fixation of the carcinogenic insult immediately prior to and during the early phase of replication of DNA in S-phase. These events are followed by a selection process for the early

stage of carcinogenesis, i.e., altered lectin agglutination profiles (Milo et al. 1981c) and expression of the early transformed phenocopy.

As pointed out by Zimmerman and Little (1981) and reported by Kapp and Painter (1979) the rate of scheduled DNA synthesis in human cells is proportional to the number of functional replicons in S at a given time. They also point out that repair of DNA damage is not extensive over the treatment period.

Following from this we hold the cells in G_1 block prior to S-phase entry in the presence of the exogenously supplied modulators which may amplify the number of replication sites and expose carcinogenic site(s) that would otherwise be masked. It may not require a permanent change in a molecular event such as that described or such as deficient DNA repair synthesis or persistence of damage, but it may require a change in events of the replicon similar to that seen in normal differentiation patterns. As we presently know, human nasopharyngeal carcinomas tumors are exceedingly difficult to grow in vitro. They purportedly differentiate in culture and produce keratin and enter an amitotic stage, (R. Glaser, pers. comm.). Milo et al. (1981a) showed that carcinogen-initiated keratinocytes when seeded directly into soft agar produce colonies. They express anchorage-independent growth. However, when carcinogen-initiated keratinocytes are transferred to culture conditions following carcinogen treatment they tend to differentiate and proceed into an amitotic stage. It is possible that there is a program for carcinogenesis in human cells that when followed rigorously can lead to a reproducible expression of a carcinogenic event. This program is comprised of a sequence of events that must occur prior to expression, i.e. activation, selective adduct formation, error-prone repair, protein modulation. These events are then followed by a program of events leading to a transformed phenocopy exhibiting anchorage-independent growth, cellular invasiveness and neoplasia.

ACKNOWLEDGMENT

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COMMENTS

CONNEY: I have heard of human cells that were transformed in vitro by chemicals, that by all criteria in vitro are transformed cells, but when they are put into nude mice, they start to grow and form a nodule, and then the nodule regresses. There is really something different about this cell. It may be a quasimalignant cell. Have you seen these results?

MILÓ: No! Not after a 4-week interval has elapsed--before tumors are counted.

CONNEY: Is there something in the environment of a so-called quasimalignant cell that can cause that cell to revert back to normal?

MILO: What is the normal phenotype that a cell should regress back towards to be described as a "normal" cell? Tumor material taken from soft-tissue tumors of humans behaves in a nude mouse system in a similar manner as that described for *in vitro* transformed cells.

PREUSSMANN: I was astonished at the vast differences in the cytotoxicity of your compounds. The nitrosoureas were less cytotoxic and the nitrosamines were very cytotoxic. You would expect the reverse order, because nitrosoureas are used in cancer chemotherapy as cytotoxic agents. Could it be that the solutions that you used in your experiments deteriorated after you used it? Those are unstable compounds. Have you any explanation for that?

HICKS: We would confirm the same observation in bladder cells. We cannot kill human bladder cells in culture with MNU.

MICHEJDA: MNNG in human fibroblasts is extremely cytotoxic. MNNG was also rather noncytotoxic in this experiment.

TANNENBAUM: Your effective concentration of NDMA is 1 ng/ml. per milliliter. Many people have done experiments with NDMA in a variety of cells. Usually they are up in millimolar concentrations before they begin to get effect. Has anyone characterized anything about the enzymology of these?

MILO: We are working on it right now.

PREUSSMANN: Could your solution of nitrosourea have been degraded after you put it into your cells?

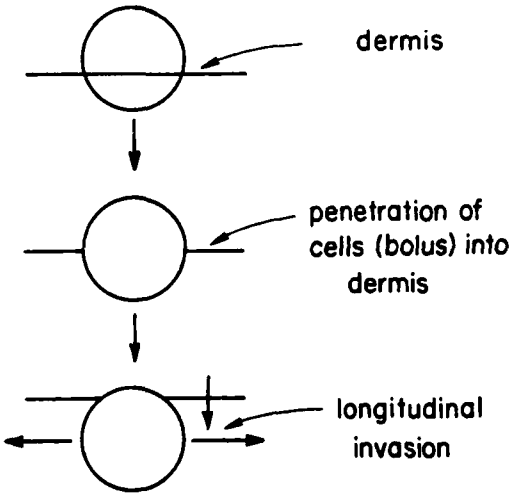
MILO: Chromatically speaking we know that what we put on does not degrade. NDMA or NDEA also appears to be stable when evaluated by HPLC.

GOLDFARB: Is there really any evidence that we are seeing real invasion or infiltration by these tumor cells into the skin?

MILO: If this occurs you occasionally will see necrosis. The suppression phenomenon can be seen by virtue of the fact that what will happen is that the epidermal type of cells, when they are put on the CES, will have this kind of feature. You will see compression all through the CES when the cells are layered on the skin. When you see invasion, what is seen when subsequently thick slices are taken is shown in the accompanying figure:

Compression of Cells (bolus)

Invasion of CES



When the slides are positive you will see the cells growing down into the CES, and longitudinally under the dermal layer of CES. So we take sequential slices in this area to try to address the problem that you are talking about.

From: BIOCHE... ...MICS OF
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ALTERATIONS IN tRNA METABOLISM AS MARKERS OF NEOPLASTIC TRANSFORMATION

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INTRODUCTION

Numerous changes in tRNA modification and catabolism are observed when cells undergo neoplastic transformation. The nature of certain of these changes is well established, while others require further characterization. Enzymes involved in the macromolecular modification of tRNA, the tRNA methyltransferases, exhibit idiosyncratic alterations during neoplastic transformation. These alterations include increases in enzyme specific activity as well as the appearance of different tRNA methyltransferases in the malignant tissue[1,2]. The increased tRNA methyltransferase activity and capacity observed *in vitro* for the enzymes isolated from transformed cells can also be correlated to increased methylation of specific tRNA isoaccepting species, although not total tRNA, *in vivo*[3].

Elevated turnover of tRNA is another characteristic of neoplastic transformation[4,5], and the increased rate of tRNA catabolism may explain the lack of extensive hypermethylation of total tRNA in malignant cells. Most modified nucleosides in tRNA cannot be salvaged when the macromolecules are degraded, and therefore, they are excreted[4]. Monitoring the elevated excretion of these tRNA catabolites by cancer patients is being investigated to determine the usefulness of these components as biochemical markers for cancer[6-8].

The enhanced generation of methylated tRNA catabolites by cancer patients may also have a fundamental role in the neoplastic process. Chronic exposure of normal mammalian cells in culture to specific methylated purine RNA catabolites can lead to neoplastic transformation[9,10]. However, the mode of action by which these natural products elicit such a response has not been established.

The appearance of many unique tRNA isoaccepting species is another common feature established for malignant cells[4,11,12]. In some cases, these species appear to differ from their normal counterparts with respect to their macromolecular modifications. Hypomodification for Y-base adjacent to the anticodon in phenylalanine tRNA and for Q-base in the first position of the anticodons for histidine, tyrosine, asparagine, or aspartic acid tRNA's is responsible for the appearance of some of the different isoacceptors in transformed cells[12-14]. Again, these tRNA aberrations offer biochemical markers for neoplastic transformation.

In this report, we examine certain of the alterations in tRNA metabolism associated with neoplasia. Potential interrelationships between the changes in tRNA modification and catabolism are explored, and a role for these aberrations in the expression of carcinogenesis is postulated.

MATERIALS AND METHODS

Nucleosides in urine were resolved and quantitated using reversed-phase high performance liquid chromatography[8] following clarification on a boronate column[15]. Quantitation was relative to the creatinine content in random urine specimens[7].

Establishment and propagation of primary cultures of Chinese hamster embryo cells were as previously described[9,16] except that the culture medium was supplemented with only 5% fetal bovine serum. These cells typically exhibit a finite lifetime in culture of 10 to 12 passages under the conditions employed. The methods for transforming these cells by chronic exposure to selected methylated purines have also been published[9,10]. The concentration of methylated purine utilized was always 10 μM.

The assay for Q-deficient tRNA makes use of the enzyme tRNA transglycosylase from Escherichia coli[14]. This enzyme can utilize mammalian tRNA's for histidine, tyrosine, asparagine, and aspartic acid as substrates only if they are hypomodified, i.e., the tRNA's have guanine in the first position of the anticodon instead of Q-base[14]. Transfer RNA from proliferating Chinese hamster cells treated with 10 μM 7-methylguanine was isolated utilizing published protocols[17], and it was evaluated as a substrate for the E. coli enzyme. The assay for Q-hypomodification involves an exchange

reaction with [8-^{3}H]guanine. Previously published methods were employed[14,18]. Yeast tRNA is Q-deficient, and therefore, was utilized as a positive control. E. coli tRNA is Q-sufficient, so it was used as a negative control. A tRNA transglycosylase from rabbit erythrocytes[19] was used to assess enzyme inhibition by 7-methylguanine.

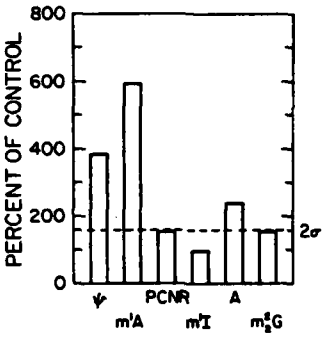

Fig. 1. Excretion of nucleoside markers by a patient at the time of of diagnosis of NPC. The results were calculated as nmoles nucleoside/μmole creatinine, and are expressed relative to normal values as percent of control. The dashed line denotes the position of two standard deviations above normal for adenosine, the marker exhibiting the largest relative standard deviation. Excretion levels above the dashed line represent significant ($P<0.02$) increases for adenosine and highly significant ($P<0.01$) for the other nucleosides. The abbreviations are: ψ, pseudouridine; m^1A, 1-methyladenosine; PCNR, 2-pyridone-5-carboxamide-N'-ribofuranoside; m^1I, 1-methylinosine; A, adenosine; and m^2_2G, N^2,N^2-dimethylguanosine.

RESULTS

Nucleoside Excretion by Cancer Patients

Urinary nucleoside excretion has been quantitated for patients with nasopharyngeal carcinoma (NPC) and leukemia, and the results

have been compared to normal excretion levels. The normal excretion values (nmoles nucleoside/μmole creatinine) used for comparison were as follows[8]: pseudouridine, 24.8; 1-methyladenosine, 2.02; 2-pyridone-5-carboxamide-N'-ribofuranoside, 1.14; 1-methylinosine, 0.96; 1-methylguanosine, 0.70; adenosine, 0.23; and N^2,N^2-dimethylguanosine, 1.05. The greatest relative standard deviation for the controls was 30.4% for adenosine.

The relative nucleoside excretion pattern for a Caucasian NPC patient at the time of diagnosis is presented in Fig. 1. At that time, the excretion levels of pseudouridine and 1-methyladenosine

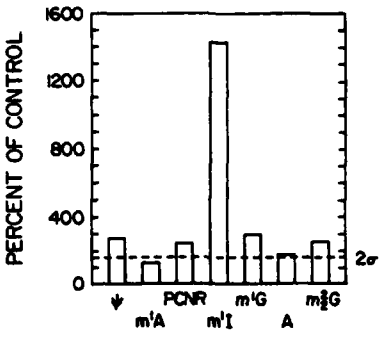

Fig. 2. Excretion of nucleoside markers by a patient at the time of diagnosis of AML. See legend to Fig. 1 for details. The additional abbreviation is: m^1G, 1-methylguanosine.

were elevated 4-fold and 6-fold respectively. Cells from tumor tissue biopsies contained the Epstein-Barr virus (EBV) genome (12.5 equivalents/cell)[20], and the patient's serum contained very high levels of antibodies to an EBV-specific DNase (10.2 units neutralized/ml serum).

The nucleoside excretion pattern for an individual at the time of diagnosis of acute myelogenous leukemia (AML) is shown in Fig. 2. In this case, 1-methylinosine was the primary marker with an increase of greater than 14-fold. Four of the other nucleosides (pseudouridine, 2-pyridone-5-carboxamide-N'-ribofuranoside, 1-methylguanosine, and N^2,N^2-dimethylguanosine) were elevated

approximately 3-fold. This patient also had significantly elevated adenosine deaminase levels in his peripheral blood cells (21.0 units/10^6 cells) compared to normal (8.4 units/10^6 cells).

Cell Transformation by Methylated Purines

The significant increase in the excretion of modified RNA catabolites by cancer patients led to the examination of the response of normal mammalian cells to these components[9,10]. Certain methylated purines were found to transform Chinese hamster embryo cells *in vitro*, with neoplastic transformation being demonstrated in some cases[10]. A summary of methylated purines evaluated and those transforming the cells for proliferative capacity (finite to continuous lifetime in culture) can be seen in Table 1. Certain of the RNA catabolites (1-methylguanine and 7-methylguanine) greatly enhance the generation of continuous cell lines, while others do not. Other naturally occurring methylated purines (7-methylxanthine and 1,3,7-trimethylxanthine) are also quite effective in transforming the cells.

The expression of various transformed phenotypes appearing during continuous exposure to the methylated purines can be reversed by removal of the methylated purine. An example showing increased saturation density of a 7-methylxanthine-transformed cell line is presented in Fig. 3. Removal of 7-methylxanthine at passage level 15, a passage level exceeding the normal number of passages obtainable before senescence and cell death, resulted in a significant decrease in the cell density of subsequent passage levels. In 3 of 4 independent transformation experiments with 7-methylxanthine, the cultures went through such a "crisis"

Table 1. Methylated Purines Generating Continuous Chinese Hamster Cell Lines

Methylated Purine	Continuous/Treated
None (Control)	2/30
Guanine (Control)	0/2
1-Methyladenine	0/2
1-Methylguanine	15/16
3-Methylguanine	0/2
7-Methylguanine	11/12
1-Methylhypoxanthine	0/2
1-Methylxanthine	1/4
3-Methylxanthine	0/2
7-Methylxanthine	4/4
1,3,7-Trimethylxanthine	4/6

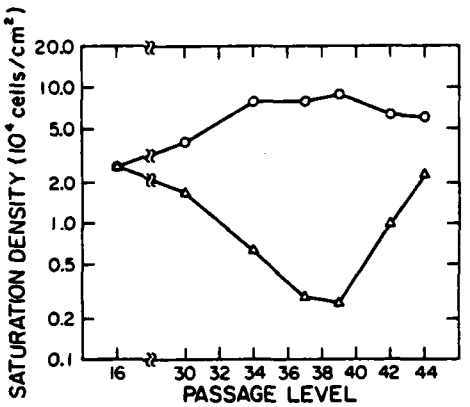

Fig. 3. Saturation densities for a 7-methylxanthine-transformed Chinese hamster cell line. The cells had been treated continuously with 10 μM 7-methylxanthine since the first passage of the primary culture. When the transformed, "continuous" cell line was subcultured at passage 15, duplicate cultures were maintained thereafter in the presence (o) or absence (Δ) of 7-methylxanthine. Saturation densities were determined after confluent cultures were split 1:4 and allowed to grow for 7 days. Duplicate cultures were trypsinized, and cells were counted with a hemacytometer.

period after removal of the methylated purine. With a 1,3,7-trimethylxanthine-transformed cell line, the cloning efficiency in soft agar decreased 6-fold after removal of the methylxanthine (unpublished observation), and tumorigenicity in nude mice was reversed by removal of 1-methylguanine from a corresponding cell line[21]. In the latter case, there was no change in the cloning efficiency in soft agar or any other in vitro characteristic related to transformation.

Q-Hypomodification of Cellular tRNA

The enzyme tRNA transglycosylase from mammalian sources catalyzes the reaction depicted in Fig. 4. Transfer RNA isolated from normal cells is mainly in the Q-modified form, while tRNA from transformed cells is Q-deficient[14]. The possibility that 7-methylguanine, a structural analog of Q-base, might inhibit Q-modification of tRNA was examined by treating normal Chinese hamster cells with 10 μM 7-methylguanine; the same concentration and conditions used for transformation. Transfer RNA was isolated from treated and untreated normal cells after 6 population doublings and assayed for Q-deficiency. Transfer RNA was also isolated from cells treated for 4 population doublings followed by no treatment for 2 more doublings to assess reversibility of any 7-methylguanine-induced Q-hypomodification. As can be seen in Table 2, 7-methylguanine induced Q-hypomodification of tRNA in the cells, and the Q-deficiency was reversible.

A tRNA transglycosylase isolated from rabbit erythrocytes was also shown to be inhibited by 10 μM 7-methylguanine *in vitro*. In 4 separate experiments with Q-deficient yeast tRNA, the percent inhibition obtained was 60.1 ± 7.6 (mean ± standard deviation) when the guanine substrate concentration was 1 μM.

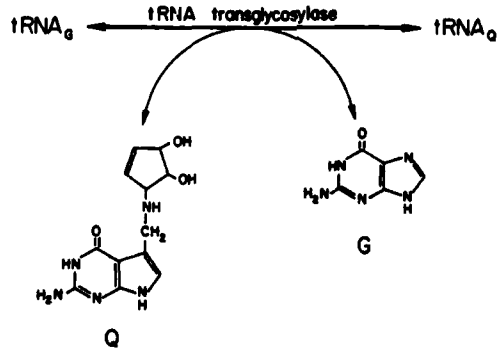

Fig. 4. tRNA transglycosylase reaction responsible for exchanging Q-base for guanine in the first position of the anticodon of tRNA's for tyrosine, histidine, asparagine, and aspartic acid. The abbreviations are: Q, 7-(3,4-*trans*-4,5-*cis*-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanine; and G, guanine.

Table 2. Q-Hypomodification of tRNA in Chinese Hamster Cells Induced 7-Methylguanine

tRNA Source	Guanine Incorporation (pmoles/hr/A_{260} unit)
Chinese hamster cells	1.86
Plus 7-methylguanine	3.46
Minus 7-methylguanine	2.09
Escherichia coli	<0.2
Yeast	6.58

DISCUSSION

The potential value of modified nucleosides as biochemical markers for cancer can be seen in Fig. 1 and Fig. 2. Even at the time of cancer diagnosis, nucleoside excretion was elevated significantly for the NPC patient (Fig. 1), and this correlated with high serum antibodies to EBV antigens including antibody to the EBV DNase, a marker for NPC[22]. The AML patient exhibited even higher nucleoside excretion levels at the time of diagnosis (Fig. 2), although the pattern of elevated excretion was different. Unique excretion patterns may offer additional means to characterize specific cancers. The patient with AML also exhibited elevated peripheral blood cell adenosine deaminase activity, another potential biological marker for leukemia. The clinical value of monitoring various markers for leukemia and NPC is being assessed for both diagnostic and prognostic purposes.

The early increases in tRNA catabolism associated with neoplasia led to an examination of the potential role of the catabolites in neoplastic transformation. The discovery that chronic exposure to some, but not all, methylated purines derived from cellular RNA can transform normal diploid cells was quite perplexing[9,10]. However, it appears that the methylated purines may influence the expression of various transformed phenotypes. Removal of the transforming methylated purine at the appropriate time can result in reversal of expression of various transformed phenotypes, e.g., increased proliferative capacity (Fig. 3), anchorage independent growth, and tumorigenicity[21]. All transformed phenotypes are not reversed by removing the methylated

purine from a particular culture. However, it was demonstrated previously that the methylated purine-transformed cells exhibit elevated tRNA methyltransferase activity[9], and therefore, the endogenous methylated purine level may negate the need for an exogenous source.

The results obtained with the methylated purines suggested similarities to dedifferentiation associated with carcinogenesis. Since dedifferentiation reportedly involves changes in gene regulation at the post-transcriptional level[23], we have attempted to identify cellular targets for the methylated purines that might alter phenotypic expression by similar means. A proposed target was tRNA transglycosylase, since the enzyme from E. coli is inhibited by the methylated purine 7-methylguanine[18]. It was presumed that a major structural change (guanine vs Q-base) in transformed tRNA's for histidine, tyrosine, asparagine, and/or aspartic acid generated by inhibiting the transglycosylase might allow the altered tRNA isoaccepting species to translate disparate mRNA's more efficiently. As we have now found, 7-methylguanine does inhibit tRNA transglycosylase from a mammalian source, and it induces Q-hypomodification of cellular tRNA (Table 2) under conditions leading to the expression of transformation.

The question of whether Q-deficient tRNA's actually have some role in the expression of transformed phenotypes remains to be answered. However, it has been reported that reversing tRNA Q-deficiency in tumor cells by administration of purified Q-base was associated with diminution of tumor cell growth in vivo[24].

The numerous alterations in tRNA metabolism associated with neoplasia have led us to devise a scheme by which they may interrelate in the expression of carcinogenesis, and the proposed sequence of events is presented in Fig. 5. The induction (initiation) of carcinogenesis could be by any means. The subsequent events are then predicted to have cause and effect relationships, i.e., each change depicted would occur in order and be caused by the previous change. Therefore, soon after the induction event there would be an increase in tRNA methyltransferase activity which would result in an increase in methylated RNA catabolites. The higher endogenous levels of methylated purines would then modulate tRNA modification by inhibiting tRNA transglycosylase. The methylated purines might also modulate tRNA modification by acting as an alternate substrate for the transglycosylase or by other, as yet unidentified, means. Both the modulation and methylation steps would be involved in generating altered tRNA isoaccepting species, and these species might allow the translation of different mRNA's that are not translated efficiently by the normal tRNA population. It is then assumed that some of these translation products would be onco-developmental proteins. If

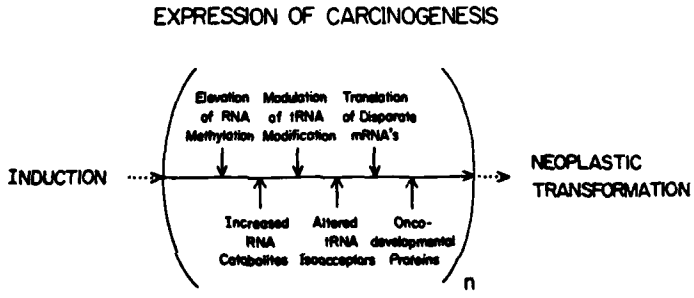

Fig. 5. Proposed model for the role of altered tRNA metabolism in the expression of carcinogenesis. Each event in the sequence is predicted to influence subsequent events, and the last may reinitiate the first.

any of these proteins were tRNA methyltransferases, and it is known that enzymes with different specificities appear[1], the cycle would repeat at a more aberrant level. By this means, the cycle could continue to generate accruing phenotypic alterations until neoplastic transformation is attained.

The proposed model offers an explanation for the general staging process of carcinogenesis. It also allows interpretation of the phenotypic reversibility phenomenon demonstrated for the exogenous methylated purines. A step back to the previous cycle might be possible by such a withdrawal, but the increased generation of endogenous methylated purines would block any further phenotypic reversion.

Certain of the individual points outlined in Fig. 5 have been proposed by other investigators to have a role in neoplastic transformation. However, linking the increased RNA catabolites to the expression of transformed phenotypes as well as the induction of tRNA hypomodification allowed us to formulate the comprehensive scheme presented. The hypothesis is being tested using a variety of model systems, and the similarities to promotion of neoplastic transformation are being studied. The numerous biochemical markers involved should greatly facilitate these investigations.

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EVALUATING TUMOR PROMOTER ACTIVITY *IN VITRO* WITH HUMAN DIPLOID FIBROBLASTS

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INTRODUCTION

Carcinogenesis is a long-term process involving the interaction of many endogenous and exogenous factors. The role of chemical pollutants in this process is complex and not well understood in most cases. The study of chemically-induced cancer can be subdivided into the stages of initiation and promotion. Classical initiation/promotion experimental protocols involve the application on mouse skin of sub-carcinogenic doses of an initiator [e.g. the polynuclear aromatic hydrocarbon (PAH) benzo(a)pyrene] followed by repeated applications of a non-carcinogenic tumor promoter [e.g. the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)]. In general, this initiation/promotion scheme *in vivo* can also be achieved *in vitro* with cultured rodent cells. The means by which phorbol esters and other agents promote carcinogenesis remains a subject of conjecture. Even in the absence of chemical carcinogen initiation *in vitro*, tumor promoters are able to induce reversible phenotypic changes in rodent cells that are characteristic of *in vitro* transformation; a phenomenon described as mimicry of transformation (1).

Although the rodent cell systems offer useful models for many studies of initiation/promotion and mimicry of transformation, the development of a human cell model would be highly desirable for the study of interactions of exogenous chemical agents in human cancer. Milo *et al.* (2) have demonstrated neoplastic transformation of normal human fibroblasts *in vitro* subsequent to treatment with complete chemical carcinogens, i.e., carcinogens that do not require promotion. However, classical initiation/promotion has not been achieved with human cells *in vitro* due to the lack of an appropriate model for the promotion stage. Diamond *et al.* (3) did report growth stimulation of normal human fibroblasts treated with TPA, but the transient 50% increase in the saturation density of TPA treated cells was attributed to a decrease in cell size induced by TPA. Few other studies on normal human cells in culture have described effects of tumor promoters that could be equated to mimicry of transformation. Tumor promoters alone do induce changes in the growth

characteristics of mutant human fibroblasts (4), and they also influence growth, differentiation, and DNA synthesis in human cancer cells (5,6,7).

Because few phorbol ester-induced effects on normal human cells have been demonstrated and those that have are not of the magnitude reported for rodent cells, it might be concluded that promotion of carcinogenesis is not relevant in man. Epidemiological studies suggest, however, that promotion is an important consideration. Therefore, we are developing a normal human cell culture system responsive to tumor promoters; one which should allow the study of mimicry of transformation as well as two-stage (initiation/promotion) carcinogenesis.

MATERIALS AND METHODS

Chemicals

The potential promoting compounds examined in this study included the phorbol esters TPA and 4-O-methyl-TPA (P.L. Biochemicals Inc., Milwaukee, Wisconsin). The former is the most potent tumor promoter on mouse skin, while the latter is an inactive structural analog (8). Mezerein, another plant diterpene and much weaker promoter than TPA on mouse skin (9), was obtained from Dr. L. David Tomei, Comprehensive Cancer Center, The Ohio State University. Other non-phorbol esters evaluated were anthralin, (1,8-dihydroxy-9-anthrone), 7,12-dimethylbenz(a)anthracene (7,12-DMBA) (both courtesy of Dr. George E. Milo, Department of Physiological Chemistry, The Ohio State University), and norharman, (9H-pyrido-[3,4-b]indole), (Sigma Chemical Co., St. Louis, Missouri). Anthralin is also a tumor promoter on mouse skin (10), while norharman, a component of tobacco smoke, is a co-mutagen (11). The PAH 7,12-DMBA is a complete carcinogen in rodent systems, but is inactive with normal human cells in vitro (12).

Cell Culture

Primary cultures of normal human fibroblasts were prepared from neonatal foreskin as described by Riegner et al. (13). The cells were grown in Eagle's minimal essential medium (GIBCO, Grand Island, New York) supplemented with 25 mM HEPES (pH 7.2), 0.2% sodium bicarbonate, 1.0 mM sodium pyruvate, gentomycin (5 µg/ml), 2X vitamins, 1X nonessential amino acids, and 2X aspartic acid, asparagine, histidine,

phenylalanine, and tyrosine. These modifications were found to enhance changes in phenotypic expression promoted by phorbol esters (manuscript in preparation), and the medium is referred to hereafter as promotion medium. The medium was also supplemented with either 20% fetal bovine serum (Reheis Chemical Co., Phoenix, Arizona, or Sterile Systems, Logan, Utah) or 20% bovine serum (Sterile Systems) for establishing primary cultures. At the first passage of the primary culture, the cells were split at a ratio of 1:4 into 25 cm^2 flasks, and the serum content of the medium was reduced to 10%. Once a week the cells were subcultured at a ratio of 1:10 and counted with a hemacytometer to determine saturation densities. Duplicate cultures were maintained for each treatment, and the cultures were fed with fresh promotion medium three days following subculture.

Each of the potential promoting agents was dissolved in acetone and added to promotion medium at a final concentration of 10^{-7}M and 0.01% acetone. For dose-response studies, promotion medium containing 10^{-6}M TPA and 0.1% acetone was prepared, from which serial dilutions were made. Control cultures were treated with the 0.01% or 0.1% acetone. Treatment of the cells with agents of interest was started at the first passage of the primary culture and continued throughout all subcultures.

DNA Synthesis

The system that we utilized to determine DNA synthesis is a modification of a procedure originated by Ball et al. (14) that allows us to grow, treat, and process human fibroblasts in glass scintillation vials. A primary culture of human fibroblasts was trypsinized and counted. Cultures were seeded by pipeting 2.0 ml aliquots of a suspension (0.25 X 10^5 cells/ml) into a series of sterile vials. When plated into the vials, promotion medium plus 20% BS was used. The cells were allowed to establish five days at which time they were confluent. The experiment was started by the addition of 10^{-7} M promoter and 0.01% acetone to the quiescent culture. Acetone alone (0.01%) was added to control cultures. To measure DNA synthesis, [^{3}H]thymidine (^{3}H-TdR) (Sp. act. 5 Ci/mmole) was added to each vial (0.5 µCi/vial) at various intervals. The cells were pulse labeled for 90 minutes and the medium was decanted.

Precursor incorporation was stopped by the immediate addition of 10 ml of ice-cold saline. The cells were gently rinsed 2 times. One ml of 1.5% (v/v) perchloric acid (PCA)

was applied to remove any unincorporated radioactive precursor and fix the cells to the surface of the vial. The acid was removed and counted to monitor any changes in the thymidine pool caused by exposure to promoter. Two further washes with 10 ml of 1.5% PCA were then applied. The vials were rinsed with 10 ml of 95% ethanol and inverted to dry. Finally, one ml of 5% PCA (v/v) was added to each vial and the samples were heated at 80°C for 40 minutes to hydrolyze nucleic acids. After cooling, scintillation fluid was added to each vial and the samples were counted.

RESULTS

Saturation Density

Significant changes in saturation density were observed when the cultured human fibroblasts were exposed continuously to TPA. The cells exhibited a loss of sensitivity to contact inhibition with an extreme degree of overgrowth. Figure 1 shows the saturation density dose response for cells treated with TPA. The saturation density for control cells was approximately 50,000 cells/cm^2 at all passages. Treatment with 10^{-8}M TPA gave the largest increase in saturation density above control values (up to 5-fold) in the experiment depicted. TPA at a concentration of 10^{-7}M was effective to a lesser degree, followed by 10^{-6} and 10^{-9}M TPA which produced nearly equal increases. A marked elevation in saturation density was observed for 10 independent primary cultures treated with 10^{-7}M TPA, with the maximum treatment values varying from 120,000 to 280,000 cells/cm^2. Substituting bovine serum for fetal bovine serum in the promotion medium had no significant effect on the TPA-induced response. Removal of TPA from the culture medium at any passage resulted in a return to near the control saturation density by the next passage.

At a concentration of 10^{-7}M, continuous treatment with 4-O-methyl-TPA, anthralin, norharman, or 7,12-DMBA had no effect on the saturation density of diploid human fibroblasts, i.e., the values were indistinguishable from the controls. However, when 10^{-7}M norharman was added to cells already being treated with 10^{-7}M TPA, a significant response was obtained (Figure 2). The norharman-induced increase was 2-fold greater than that induced by TPA alone, and it was transient in nature. It has not been possible to obtain similar effects when TPA and norharman were added concurrently at the first passage of the primary culture. In

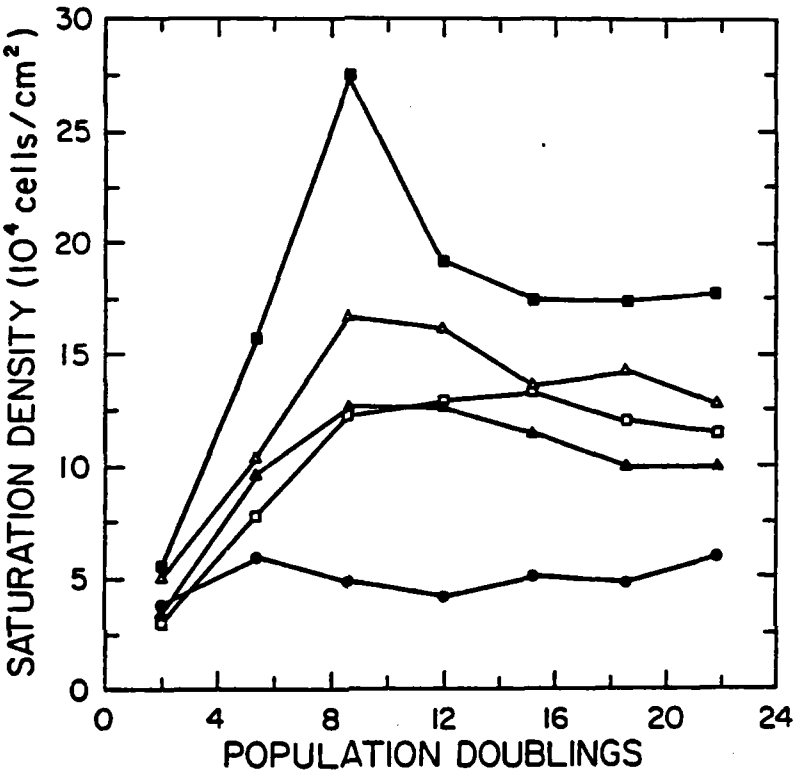

FIGURE 1. TPA dose-response curve. Human fibroblasts treated from passage one with TPA. The concentrations utilized were 10^{-6}M (▲), 10^{-7}M (△), 10^{-8}M (■), and 10^{-9}M (□). Control cells (●) were treated with 0.1% acetone.

a repeat of the experiment depicted in Figure 2, removal of norharman at any time after the synergistic increase in saturation density, resulted in a return to the TPA density by the subsequent passage.

Anthralin at a concentration of 10^{-7}M was also found to elevate the saturation density attainable in concert with 10^{-7}M TPA (Figure 3). As described earlier, 10^{-7}M anthralin alone was without effect. As shown in Figure 3, 10^{-7}M mezerein also promoted an increase in growth beyond

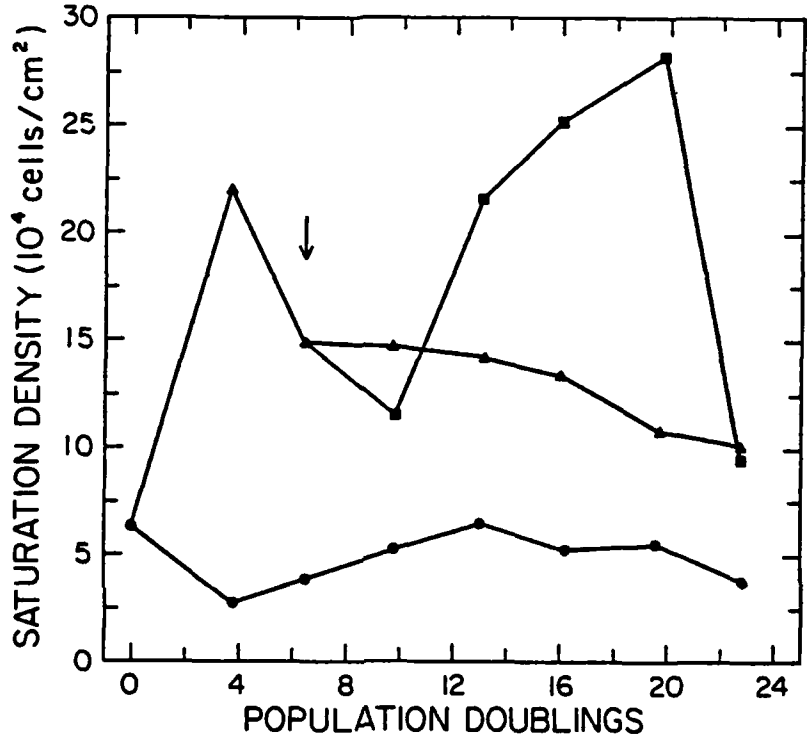

FIGURE 2. Effects of TPA plus norharman on saturation density. Cells were exposed continuously to 10^{-7}M TPA (▲) and after two passages to 10^{-7}M norharman (■). Control cells (●) were treated with 0.01% acetone. The arrow indicates the point at which norharman was added to duplicate cultures.

that obtained with 10^{-7}M TPA. However, continuous treatment with 10^{-7}M mezerein alone causes a reversible 2 to 3-fold increase in the saturation density of the normal human cells, so the effects of TPA plus mezerein may be additive.

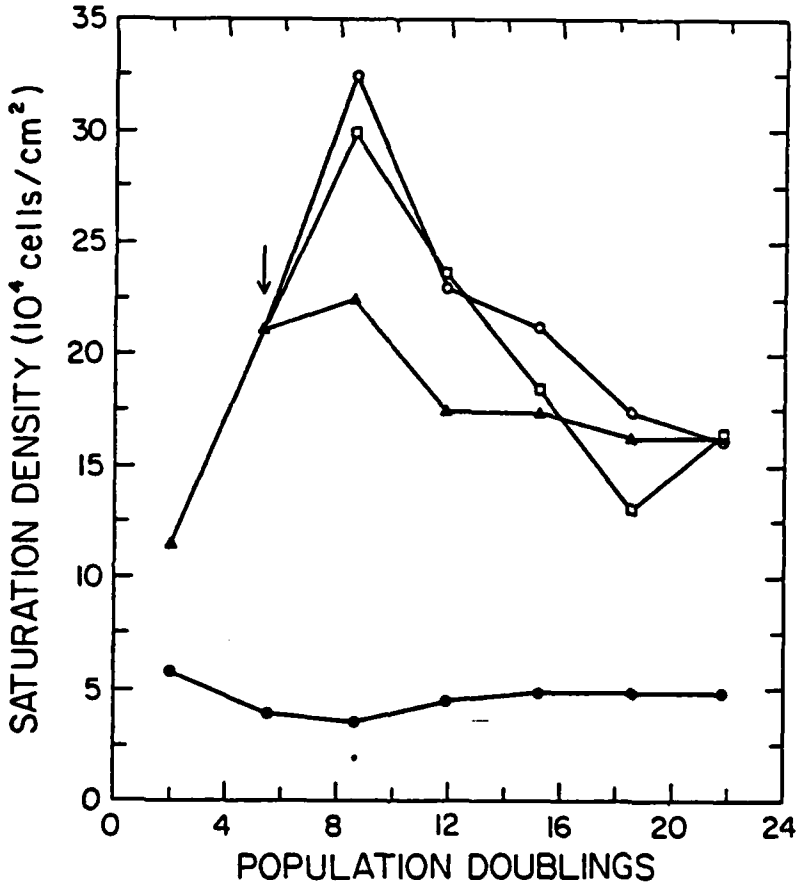

FIGURE 3. Effects of TPA plus anthralin or mezerein on saturation density. Addition of 10^{-7}M anthralin (□), or 10^{-7}M mezerein (○) to promotion medium of cells already exposed to 10^{-7}M TPA (▲) for three passages. Control cells (●) were treated with 0.01% acetone. The arrow indicates the point at which anthralin and mezerein were added to duplicate cultures.

DNA Synthesis

Changes in DNA synthesis were also observed with human skin fibroblasts treated with TPA (Figure 4). There was an initial inhibition of ^{3}H-TdR incorporation followed by a large stimulation of incorporation. TPA at a concentration of 10^{-7}M (the concentration depicted) gave the greatest and most prolonged inhibition of DNA synthesis, followed by 10^{-8} and 10^{-9}M. The DNA stimulatory response was nearly equal for 10^{-7} and 10^{-8}M TPA and was undetected for 10^{-9}M TPA. However, the TPA-induced stimulatory (mitogenic) response was less reproducible and more influenced by culture conditions than the inhibitory response. Treatment of human diploid fibroblasts with 10^{-7}M 4-O-methyl-TPA, norharman, anthralin, or 7,12-DMBA had no effect on DNA synthesis. Treatment with norharman or anthralin in conjunction with TPA also had no effect on the inhibition or stimulation of DNA synthesis caused by TPA. Mezerein at a concentration of 10^{-7}M inhibited DNA synthesis in a transient manner, similar to TPA.

DISCUSSION

It was established in this investigation that under appropriate culture conditions continuous exposure to TPA or mezerein significantly alters phenotypic and biochemical properties of low passage human diploid fibroblasts. An acute TPA-induced mitogenic response similar to that depicted in Figure 4 has been demonstrated previously with normal human cells (15), but reported phenotypic changes for these cells are minimal (3). The 50% increase in saturation density induced by TPA in the previous studies was attributed to a somewhat smaller cell size (3). Even then, a 50% increase above the normal cell density for human cells of 40,000 to 50,000 cells/cm^2 would yield only 60,000 to 75,000 cells/cm^2. That is a hardly comparable to levels up to 280,000 cells/cm^2 demonstrated under the conditions prescribed in this investigation. Although a significant elevation in saturation density was induced by TPA, some variability was observed in the magnitude and timing of the response. In addition, the secondary response to norharman, anthralin, and mezerein in TPA treated cultures was greatest when the cells were highly responsive to TPA. This variability suggests differences in promoter sensitivity among primary human skin cell cultures; an aspect currently being explored.

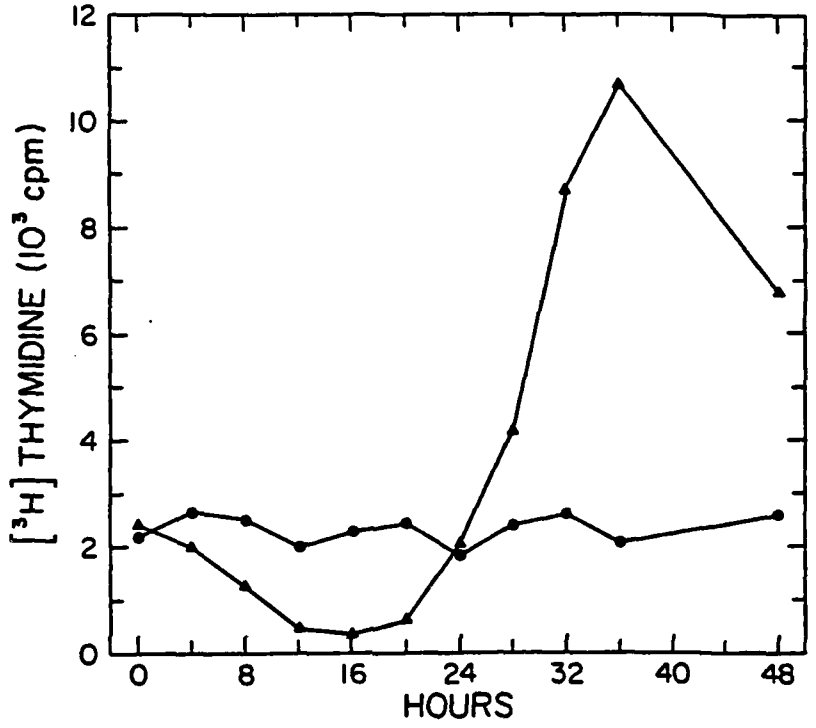

FIGURE 4. Effects of TPA on DNA synthesis. Primary cultures of normal human fibroblasts were seeded and grown in glass scintillation vials (Materials and Methods). Five days later the confluent, quiescent cultures were treated with 10^{-7}M TPA (▲) or 0.01% acetone (●) at time zero. At 4 hour intervals the cells were pulse labeled with ^{3}H-TdR.

The only two agents that were able to induce mimicry of transformation by themselves, TPA and mezerein, were also the only two that inhibited DNA synthesis immediately after exposure. Peterson et al. (16) suggested that the transient inhibition of DNA synthesis in cultured mouse fibroblasts correlated with promoter activity of various phorbol esters; an observation in agreement with our human fibroblast results. Specificity is indicated by the fact that 4-O-methyl-TPA, anthralin, norharman, or 7,12-DMBA alone neither inhibited DNA synthesis nor promoted a loss of

contact inhibition of growth. Therefore, evaluating agents for their ability to inhibit DNA synthesis in human diploid fibroblasts following acute exposures may offer a rapid means to identify "complete promoters" for these cells.

The effects of norharman and anthralin in conjunction with TPA are more complex. Apparently these compounds are "incomplete promoters" or "co-promoters" for inducing mimicry of transformation of normal human cells. The promotion of mouse skin tumors was reported by Slaga et al. (17) and Furstenberger et al. (18) to be at least a two-stage process, with some agents functioning as second stage promoters following TPA exposure. Our in vitro human cell results are consistent with anthralin and norharman being second stage promoters, but other possibilities have not been totally ruled out. Mezerein may function both as a weak complete promoter and a second stage promoter.

The biochemical and phenotypic changes induced in human diploid fibroblasts by putative tumor promoters may aid in establishing agents which are relevant to human carcinogenesis. In addition to further investigations of mimicry of transformation, conditions are being defined to examine two-stage carcinogenesis with the human cells in vitro. By this means we hope to better understand the role of exogenous chemical agents in cancer.

ACKNOWLEDGEMENTS

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Inosine Biosynthesis in Transfer RNA by an Enzymatic Insertion of Hypoxanthine*

(Received for publication, August 29, 1983)

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An enzyme was discovered which incorporates hypoxanthine into mature tRNA macromolecules. This enzyme is postulated to be similar to tRNA-guanine ribosyltransferase which inserts 7-(3,4-*trans*-4,5-*cis*-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanine into the first position of the anticodon of four tRNAs. The hypoxanthine-incorporating enzyme has been assayed in extracts of rat liver and cultured human leukemia cells and it has been resolved from tRNA-guanine ribosyltransferase by DEAE-cellulose column chromatography. The enzyme assay is based on the incorporation of radiolabeled hypoxanthine into unfractionated heterologous tRNA and the reaction rate is proportional to the amount of added enzyme extract. Hydrolysis of the radiolabeled tRNA and analysis of the nucleoside composition yields inosine (the nucleoside of hypoxanthine) as the only radiolabeled product. It is proposed that the enzyme, a tRNA-hypoxanthine ribosyltransferase, is responsible for the biosynthesis of inosine in the anticodon wobble position of specific tRNAs, resulting in greatly expanded codon recognition by these tRNAs.

Extensive post-transcriptional modification of tRNA generates macromolecules containing an array of altered purines and pyrimidines. One modified nucleoside found exclusively in the anticodon of tRNAs is inosine. Inosine has been shown to occur in the first position of the anticodon of tRNA isoacceptors accommodating eight different amino acids, *i.e.* some of the tRNA isoacceptors for alanine, arginine, isoleucine, leucine, proline, serine, threonine, and valine contain inosine (1). The wobble hypothesis of codon-anticodon pairing proposed by Crick (2) states that inosine in the first position of the anticodon of tRNAs could base pair with uridine, cytidine, or adenosine in the third position of appropriate codons in mRNA, while standard base pair interactions would be maintained in the other two positions. Adenosine in the tRNA wobble position would base pair only with uridine, guanosine with cytidine or uridine, cytidine with guanosine, and uridine with adenosine or guanosine (2). Therefore, inosine in the anticodon expands the codon recognition potential of a particular tRNA.

The precise details of inosine biosynthesis in tRNA have not been established. Kammen and Spengler (3) demonstrated that inosine must be generated as a post-transcriptional modification to the tRNA macromolecule, but an enzyme capable of carrying out this modification reaction has not been described. Kammen and Spengler (3) could find no evidence for a postulated tRNA anticodon-specific adenosine deaminase generating inosine in the macromolecule, so it was necessary to investigate alternate biosynthetic mechanisms.

The only macromolecular purine modification which has been shown to occur by direct base replacement is the one involved in generating the nucleoside queuosine[1] in the anticodon wobble position of tRNAs for aspartic acid, asparagine, histidine, and tyrosine (4–7). The enzyme tRNA-guanine ribosyltransferase (EC 2.4.2.29) catalyzes the exchange of guanine in the primary transcript for the highly modified base queuine, thereby giving rise to the nucleoside queuosine. Evidence is presented here that inosine biosynthesis in tRNA occurs by a similar mechanism, whereby hypoxanthine is inserted directly into tRNA macromolecules.

EXPERIMENTAL PROCEDURES

Enzyme Assays—The assay conditions for tRNA-hypoxanthine ribosyltransferase were a modification of those of Howes and Farkas (5) for tRNA-guanine ribosyltransferase. The standard reaction mixture contained 10 μmol of Tris-HCl (pH 7.4), 53 μmol of KCl, 2 μmol of $MgCl_2$, 0.2 μmol of EDTA, 5 μmol of 2-mercaptoethanol, 6 nmol of allopurinol, 1.0 A_{260} unit of *Escherichia coli* tRNA, 1 μCi of [8-^{3}H] hypoxanthine (1 Ci/mmol), and enzyme extract in a total volume of 0.6 ml. The reaction mixtures in triplicate were incubated at 37 °C for 30–60 min. Radiolabeled tRNA was precipitated with ice-cold 5% trichloroacetic acid and collected on glass fiber filters for scintillation counting (8). Similar assay conditions were employed for tRNA-guanine ribosyltransferase, but with yeast tRNA and [8-^{3}H]guanine as substrates and without the xanthine oxidase inhibitor allopurinol (5, 8).

DEAE-cellulose Column Chromatography—Conditions utilized were similar to those of Shindo-Okada *et al.* (9) for rat liver tRNA-guanine ribosyltransferase. Livers excised from two Sprague-Dawley rats were immediately homogenized at 4 °C in 4–5 volumes of 10 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol, and 10% glycerol. The homogenate was centrifuged at 30,000 × *g* for 20 min at 4 °C and the resulting supernatant was then centrifuged at 105,000 × *g* for 60 min at 4 °C. The final supernatant was loaded onto a DEAE-cellulose column (25 × 2.4 cm) equilibrated with the Tris-HCl buffer described above. The column was washed with 200 ml of buffer and then was eluted with a 200-ml linear gradient of 0–0.6 M NaCl in Tris-HCl buffer. Fractions (5 ml) were collected and aliquots of 0.1 ml were assayed for tRNA ribosyltransferase activities.

Cultured Cells—Human promyelocytic leukemia (HL-60) cells, obtained from Dr. Robert Gallo at the National Cancer Institute, were grown in suspension culture in RPMI-1640 medium supplemented

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[1] The abbreviations used are: queuosine, the ribonucleoside of queuine; queuine, 7-(3,4-*trans*-4,5-*cis*-dihydroxyl-1-cyclopenten-3-ylaminomethyl)-7-deazaguanine; HPLC, high performance liquid chromatography.

with 15% fetal bovine serum. Human leukemic T-lymphoblasts (CCRF-CEM) in suspension culture were grown in Eagle's minimal essential medium supplemented with 10% bovine serum. Cells harvested by centrifugation were homogenized in 2–4 volumes of Tris-HCl buffer (described above). The homogenate was centrifuged at 30,000 × *g* for 30 min at 4 °C and the supernatant was utilized in the enzyme assays.

HPLC of tRNA Nucleosides—The enzyme assay was performed as described above, but with 5 A_{260} units of *E. coli* tRNA, 2 μCi of [8-^{3}H]hypoxanthine, and 0.1 mg of protein from an HL-60 cell extract. After a 60-min reaction time, the tRNA-hypoxanthine ribosyltransferase reaction was terminated by the addition of 3 volumes of ice-cold 50 mM sodium acetate buffer (pH 4.5) containing 5 mM 2-mercaptoethanol, 5 mM EDTA, 10 mM $MgCl_2$, and 0.3 M NaCl. The radiolabeled tRNA was isolated by DEAE-cellulose column chromatography (10) and was recovered by ethanol precipitation. The precipitate was dried *in vacuo* and redissolved in 50 μl of water. The tRNA was then subjected to enzymatic hydrolysis with *Penicillium citrinum* nuclease P1 and *E. coli* alkaline phosphatase as described by Gehrke *et al.* (11) and was analyzed for radiolabeled nucleoside content by reversed phase HPLC (12). The column used was an IBM-ODS (4.5 × 250 mm) with an initial mobile phase of 94.5% 0.1 M $NH_4H_2PO_4$ (pH 5.1) and 5.5% methanol. After 18 min, the methanol concentration was increased to 8.5%. Fractions were collected and counted by liquid scintillation.

Materials—*E. coli* tRNA was obtained from Biogenics Research Corp., Chardon, OH, while yeast tRNA and *P. citrinum* nuclease P1 were obtained from Boehringer Mannheim. *E. coli* alkaline phosphatase was obtained from Sigma. The radiolabeled purines [8-^{3}H]hypoxanthine and [8-^{3}H]guanine were both purchased from Amersham. Fetal bovine and bovine serum were obtained from Sterile Systems, Logan, UT, while the cell culture media RPMI-1640 and Eagle's minimal essential medium were purchased from GIBCO, Grand Island, New York. The IBM-ODS HPLC column was purchased from IBM Instruments, Inc., Wallingford, CN.

RESULTS

An enzymatic activity was detected in rat liver and cultured human leukemia cells (CCRF-CEM and HL-60) which incorporates hypoxanthine into unfractionated tRNA. The rat liver enzyme was resolved from tRNA-guanine ribosyltransferase by DEAE-cellulose column chromatography (Fig. 1). The tRNA-guanine ribosyltransferase eluted at a NaCl concentration of approximately 0.04–0.06 M as previously reported by Shindo-Okada *et al.* (9), while the putative tRNA-hypoxanthine ribosyltransferase eluted at a concentration of 0.25–0.30 M NaCl (Fig. 1). The latter enzyme is very labile, with a half-life *in vitro* of only a few hours, making further purification difficult. Limited cross-reactivity between substrates was exhibited by the enzymes, with the early eluting tRNA-guanine ribosyltransferase demonstrating substrate preferences *in vitro* for yeast tRNA and guanine as previously reported (9), while the later eluting enzyme preferred *E. coli* tRNA and hypoxanthine (Fig. 1).

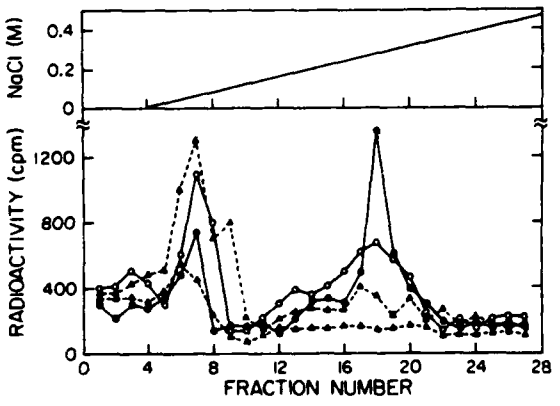

FIG. 1. **Chromatography of rat liver tRNA ribosyltransferases on a DEAE-cellulose column.** The chromatography and assay conditions were as described under "Experimental Procedures." The *symbols* indicate: *E. coli* tRNA and [8-^{3}H]hypoxanthine (●), yeast tRNA and [8-^{3}H]hypoxanthine (○), *E. coli* tRNA and [8-^{3}H]guanine (▲), and yeast tRNA and [8-^{3}H]guanine (△).

The product of hypoxanthine insertion into *E. coli* tRNA was established using the enzyme from human promyelocytic leukemia (HL-60) cells. After radiolabeling with [8-^{3}H]hypoxanthine, the tRNA was isolated and subjected to enzymatic hydrolysis. The nucleoside composition was then evaluated by HPLC (Fig. 2). Inosine was the only radiolabeled product obtained, thereby demonstrating a covalent modification to the macromolecule. Similar results were obtained with the enzyme from rat liver, but the liver enzyme studies were more difficult due to the high level of enzymes capable of metabolizing the substrates.

The rate of the hypoxanthine insertion reaction was proportional to the amount of added enzyme extract as shown with the enzyme from human leukemic T-lymphoblasts (CCRF-CEM) (Fig. 3). In addition, the time course of the reaction was linear for 30–40 min with the CCRF-CEM and HL-60 enzymes and for 60–90 min with the rat liver enzyme (data not shown).

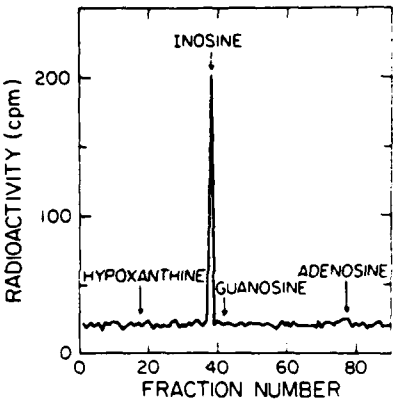

FIG. 2. **HPLC elution profile of hydrolyzed *E. coli* tRNA radiolabeled with [8-^{3}H]hypoxanthine.** Cultured HL-60 cells were used as a source of the enzyme. The enzyme assay, isolation and hydrolysis of the tRNA, and chromatography conditions were as described under "Experimental Procedures." The elution positions of authentic purine nucleosides and hypoxanthine are indicated.

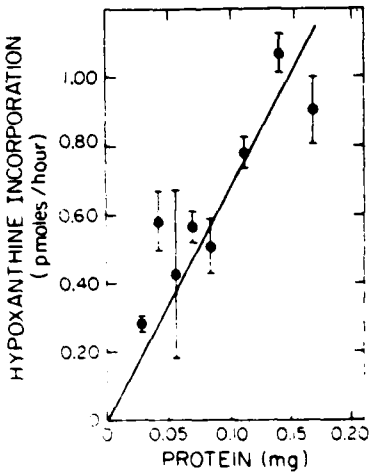

FIG. 3. **Relationship of reaction rate to enzyme protein concentration.** Cultured CCRF-CEM cells were used as the source of the enzyme as described under "Experimental Procedures." The mean of triplicate assays is indicated at each protein concentration.

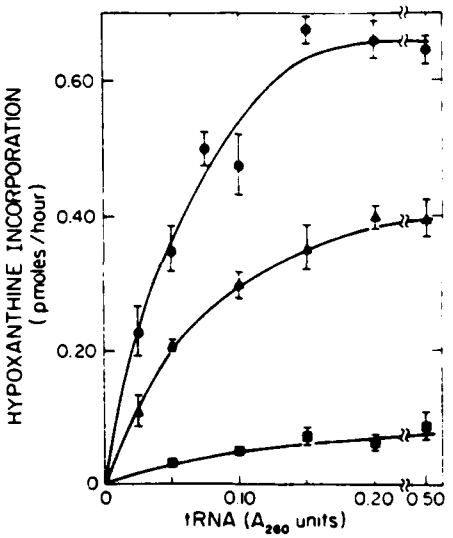

FIG. 4. **Hypoxanthine insertion into tRNA.** Cultured CCRF-CEM cells were used as a source of the enzyme. The assay conditions were as described under "Experimental Procedures" with 0.03 mg of protein from the leukemia cell extract per assay. The mean of triplicate assays is indicated for: *E. coli* tRNA (●), yeast tRNA (▲), and CCRF-CEM leukemia cell tRNA (■). CCRF-CEM tRNA was purified as described by Katze and Farkas (10).

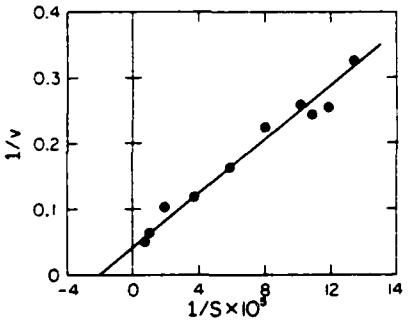

FIG. 5. **Lineweaver-Burk analysis for tRNA-hypoxanthine ribosyltransferase.** Cultured CCRF-CEM cells were used as a source of the enzyme. The assay conditions were as described under "Experimental Procedures," with hypoxanthine concentrations from 0.75–15.75 μM. The mean of triplicate assays is indicated at each point.

The hypoxanthine accepting ability of various unfractionated tRNA substrates for the CCRF-CEM enzyme are indicated in Fig. 4. *E. coli* tRNA was a better substrate than yeast tRNA for the human leukemia cell enzyme (Fig. 4) as it was for the rat liver enzyme (Fig. 1). The need for a heterologous tRNA substrate is also demonstrated in Fig. 4, since the leukemia cell enzyme was unable to insert appreciable hypoxanthine into the homologous leukemia cell tRNA.

Kinetic evaluations of the CCRF-CEM enzyme in a crude cell extract yielded a K_m for hypoxanthine of approximately 5 μM (Fig. 5). The hypoxanthine insertion reaction appeared to be irreversible, in that hypoxanthine, adenine, guanine, cytosine, and uracil were incapable of exchanging the radiolabeled hypoxanthine out of the *E. coli* tRNA (data not shown).

DISCUSSION

Examination of the genetic code indicates which codons should permit inosine wobble with their tRNA counterparts. To ensure fidelity of gene expression, only codons where uridine, cytidine, and adenosine in the third position all specify the same amino acid should be able to base pair with inosine-containing tRNAs. These codon families include those for the amino acids alanine, arginine, glycine, isoleucine, leucine, proline, serine, threonine, and valine, and, with the exception of glycine, these are exactly the same amino acids accommodated by tRNAs known to contain inosine in the anticodon wobble position (1). Therefore, the enzyme (or enzymes) responsible for the biosynthesis of inosine in the anticodon influences a broad spectrum of the genetic code.

The enzyme described in this investigation gives rise to inosine in tRNA by the covalent insertion of preformed hypoxanthine (Fig. 2). The only other tRNA modification enzyme known to insert a preformed base into tRNA is tRNA-guanine ribosyltransferase (4–7). Although that enzyme will catalyze a guanine for guanine exchange reaction *in vitro* (5), the normal function of the mammalian tRNA-guanine ribosyltransferase appears to be an exchange of guanine in the tRNA primary transcript for the modified base queuine (9). The nucleoside queuosine, like inosine, is found only in the first position of the anticodon of tRNAs (1).

That the queuosine and inosine modification reactions are catalyzed by different enzymes was established during this investigation. The queuine insertion enzyme and the hypoxanthine insertion enzyme were resolved by DEAE-cellulose column chromatography and the two enzymes were shown to have different substrate specificities *in vitro* (Fig. 1). In addition to the preferential use of hypoxanthine over guanine, *E. coli* tRNA was a preferred substrate for the putative tRNA-hypoxanthine ribosyltransferase *in vitro*. Unfractionated *E. coli* tRNA reportedly contains 60% less inosine than yeast tRNA (3) and this correlates quite well to the greater degree of hypoxanthine incorporation into *E. coli* tRNA than yeast tRNA (Fig. 4). In comparison, yeast tRNA is totally queuine-deficient while *E. coli* tRNA is queuine-modified, so the former is a much better substrate for the tRNA-guanine ribosyltransferase (9).

The need for a heterologous tRNA substrate *in vitro*, as demonstrated for the hypoxanthine insertion enzyme from human leukemia cells (Fig. 4), is as reported for other tRNA modification reactions (13). Since the homologous tRNAs are already fully modified *in situ* by the connate enzyme(s), they are poor substrates *in vitro*.

Preliminary kinetic analyses of the irreversible hypoxanthine incorporation into unfractionated *E. coli* tRNA yielded a K_m of approximately 5 μM for hypoxanthine using a crude cell extract from cultured human leukemia cells (Fig. 5). This value is comparable to the apparent K_m for guanine of 25 μM reported originally by Farkas and Singh (4) for the tRNA-guanine ribosyltransferase from rabbit erythrocytes. However, with purification of the guanine-inserting enzyme, the affinity constant for guanine was found to be approximately 100-fold lower (5), apparently due to the removal of enzymes competing for the substrates of the reaction *in vitro*. Similar results might be obtained after purification of the extremely labile hypoxanthine insertion enzyme as well.

The identify of the base in the primary transcript being exchanged for hypoxanthine remains to be established. However, reports of microinjecting cloned tRNA genes (14) or anticodon reconstructured tRNAs (15) into frog oocytes indicate that adenosine is being modified to inosine. In addition, with over 250 tRNAs sequenced to date (1), none contain adenosine in the first position of the anticodon, thereby further suggesting a hypoxanthine for adenine exchange. However, other base exchange possibilities are also being

investigated with the rat liver and human leukemia cell tRNA modification enzymes.

Regardless of which base in the primary transcript is being replaced, inosine in the first position of the anticodon will greatly expand the wobble capability of the tRNA (2). Therefore, the newly discovered tRNA-hypoxanthine ribosyltransferase could be of importance in regulating codon recognition, and, based on the number of codons potentially involved, this could have a major impact on gene expression.

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ALTERED GROWTH PROPERTIES OF NORMAL HUMAN CELLS INDUCED BY PHORBOL 12,13-DIDECANOATE

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SUMMARY

The tumor promoter phorbol 12,13-didecanoate (PDD) significantly altered the growth properties of early passage normal human skin cells in vitro in culture medium supplemented with elevated concentrations of selected amino acids. Continuous treatment of cells with 10^{-7} or 10^{-8} *M* PDD resulted in a 5 to 10-fold increase in saturation density at early passages followed by a long-term two- to fourfold increase. The PDD-treated cultures remained in exponential growth at cell densities greater than 10-fold higher than the control cultures. Removal of PDD from the culture medium while the cells were at a high cell density resulted in a return to near-normal saturation density by the subsequent passage. Anchorage independent growth of normal human cells in methylcellulose was also promoted by PDD in a dose dependent manner, with prior subculturing in the presence of PDD being required for maximal colony formation. The structural analog 4α-phorbol 12,13-didecanoate failed to elicit similar cellular responses.

Key words: tumor promoters; human skin cells; mimicry of transformation.

INTRODUCTION

Investigations of staging of carcinogenesis, based on the two-stage mouse skin model (1), have been reported with various cell culture systems (2–7). Tumor promoters elicit numerous changes in cell growth characteristics in these systems even in the absence of initiation of carcinogenesis; an in vitro phenomenon referred to as mimicry of transformation (8). However, there have been few reports of phorbol ester tumor promoters significantly altering the growth properties of normal human cells in vitro, thereby greatly impeding the study of promotion of carcinogenesis as it relates specifically to man. A slight decrease in the size of diploid human fibroblasts induced by phorbol esters was reported to allow a marginal increase (up to 50%) in the saturation density attainable under standard cell culture conditions (9). At low cell densities, phorbol esters reportedly inhibit the proliferation of normal human cells, which is in contrast to the expected in vitro response for a tumor promoter (10). The tumor promoters do induce various changes in the in vitro growth characteristics of mutant human fibroblasts and human cancer cells that are more characteristic of promotion as defined for cultured rodent cells (10–14).

The expression of transformed phenotypes by human skin fibroblasts subsequent to treatment with chemical or physical carcinogens was reported by Milo et al. (15) to be augmented by supplementing the culture medium with excess (8X) nonessential amino acids. While examining the long-term effects of the tumor promoter phorbol 12,13-didecanoate (PDD) on normal human cells in culture, it was observed that significant changes in phenotypic expression (e.g., increased saturation density) were obtained in the same medium. Partial characterization of PDD-induced changes in the growth properties of diploid human cells in vitro is the subject of this report.

MATERIALS AND METHODS

Cell culture. Primary cultures of normal human cells were prepared from neonatal foreskin as described by Riegner et al. (16), with each primary indicating an individual donor. At the

first passage of the primary culture, the fetal bovine serum (Reheis, Kankakee, IL, or Sterile Systems, Logan, UT) content in Eagle's minimal essential medium (MEM) (GIBCO, Grand Island, NY) was reduced from 20 to 10%, and it was maintained at that level for subsequent passages. The MEM was also supplemented with 25 m*M* HEPES buffer (pH 7.2), 2 m*M* glutamine, 1 m*M* sodium pyruvate, gentamicin (50 μg/ml), 0.2% sodium bicarbonate, 2X vitamins, and either 8X nonessential amino acids or 1X nonessential amino acids plus 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine. In some experiments (not described), fibroblasts and epithelial cells were separated by selective trypsinization, as described by Milo et al. (17), so each cell population could be treated individually with the phorbol ester. However, this step was not included routinely, because it was important to add the phorbol ester to the cells as early as possible. In addition, only the fibroblasts, which predominate in the primary culture, gave phorbol ester-induced responses characteristic of mimicry of transformation while epithelial cell growth (stratification) was inhibited. Addition of phorbol esters to matched cultures was initiated at the first passage of the primary culture for all experiments described. Postconfluent cell populations in duplicate were trypsinized, and cells were counted with a hemocytometer to establish saturation densities. Cell volume distribution data for phorbol ester treated and untreated cells were obtained and processed with a Coulter counter Model ZBI equipped with a C-1000 Channelyzer and Accucomp computer interface.

For the determination of anchorage independent growth, cell culture dishes (60 mm) were overlaid with 5 ml of 1.0% Difco agar in MEM supplemented with 20% fetal bovine serum, 20% tryptose phosphate broth, 1 m*M* sodium pyruvate, 1X nonessential amino acids, 2X vitamins, and 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine. The cells (10^3) in single cell suspensions were overlaid in duplicate in 10 ml of 1.3% methylcellulose in S-MEM (MEM for suspension culture) supplemented as above, but without tryptose phosphate broth. Colonies were scored after 21 d.

Phorbol esters. Phorbol 12,13-didecanoate (PDD) and 4α-phorbol 12,13-didecanote (4α-PDD) (P-L Biochemicals, Milwaukee, WI) were dissolved in acetone. The acetone concentration was 0.01% in phorbol treated and untreated cultures.

Results

Increased saturation density. The saturation density of normal human cells cultured in the medium containing 8X nonessential amino acids was significantly increased by 10^{-7} *M* PDD (Fig. 1 *A*). The saturation density for the normal control cells was approximately 4×10^4 cells cm^{-2} at most passages, whereas the PDD treated cells had saturation densities two- to fourfold higher than controls at each passage over most of the lifetime of the cultures. The specificity of the effect for a tumor promoting phorbol ester is demonstrated by the fact that 4α-PDD gave results similar to the untreated controls. PDD has induced comparable elevations in saturation density at successive passages in four different primary cultures in medium supplemented with 8X nonessential amino acids. This two- to fourfold increase in saturation density was obtained in spite of the fact that 10^{-7} *M* PDD reduced the cloning efficiency 20 to 30% with all primary cultures under the conditions employed. Lower PDD concentrations (10^{-8} *M* to 10^{-10} *M*) had no effect on the cloning efficiency of human skin cells (data not presented).

While examining the effects on saturation density of supplementation with various other combinations and concentrations of amino acids, an even greater initial increase was observed for normal cells treated with 10^{-7} *M* PDD in medium containing 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine (Fig. 1 *B*). Cell densities in the PDD treated cultures reached levels seven times those of the acetone and 4α-PDD controls in the experiment depicted, and removal of PDD from the culture medium resulted in a return to near-normal saturation density within a few population doublings (Fig. 1 *B*). Similar results have been obtained with 10 different primary cultures treated with 10^{-7} or 10^{-8} *M* PDD, with the early increases in saturation density ranging from 5- to 10-fold. Eliminating the feeding step after 3 d as described in Fig. 1 *B* or substituting bovine serum for fetal bovine serum in the culture medium for the duration of the experiment did not alter the results significantly. Although the timing of the transient increase in density has been somewhat variable from one primary culture to another, all the cultures have exhibited a long-term, two- to fourfold elevation subsequently, similar to that seen in 8X nonessential amino acids. Two of the ten primary cultures evaluated in the 2X supplemented medium also had PDD added after the

control cells had undergone 15 to 20 population doublings in culture, and under these circumstances only twofold increases in density were obtained. Both of these primaries had yielded saturation densities greater than fivefold above the untreated control cells when the phorbol ester was added at the 1st passage.

The increased saturation density induced by PDD can be seen in Fig. 2. The PDD treated cells (Fig. 2 *B*) exhibited an extreme degree of overgrowth, whereas the control (Fig. 2 *A*) and 4α-PDD treated cells (Fig. 2 *C*) did not, i.e. the latter were responsive to density dependent growth inhibition. The overgrowth of the cells treated with PDD was still readily apparent even though the saturation density attained had decreased from seven times the control on the previous passage to only three times when the photomicrographs were taken (Fig. 1 *B*). The cells in treated and untreated cultures retained a fibroblastlike morphology predominantly, but the PDD treated cells did seem smaller by microscopic observation at both low and high cell densities. Cell sizing measurements for PDD treated and untreated human cells gave results for PDD-induced decreases in cell volume ranging from negligible to 40% depending on the phorbol ester concentration, duration of treatment, and cell growth phase. Under the conditions depicted in Fig. 2 *B*, cell size was decreased 30 to 40% by PDD.

The degree to which PDD caused a loss of sensitivity to contact inhibition of cell division of normal human cells is demonstrated in Fig. 3. Cells treated with 10^{-8} *M* PDD remained in exponential growth at significantly (>10-fold) higher cell densities than untreated cells. The slight decrease in the PDD treated cell density after Day 6 in the experiment depicted (Fig. 3) was associated with the appearance of floating, nonviable cells. No further increase in dead cells or decrease in density was then observed after 7 to 8 d, i.e. the cells had entered the stationery, nonproliferating phase. The rapid cessation of growth by the PDD treated cells depicted in this experiment (Fig. 3) together with the obvious lack of density dependent inhibition of growth (Fig. 2 *B*) suggests that some essential nutrient may become limiting in the culture environment.

Anchorage independent growth. If the normal human skin cells were exposed to PDD continuously under the prescribed conditions, anchorage independent growth was also promoted in a dose dependent fashion (Fig. 4). As shown, few colonies were obtained when the cells were passaged only twice (ca 7 population doublings) with PDD before seeding in methylcellulose, whereas the number increased significantly after four passages (ca 14 population doublings). However, no further increase in the number of colonies was obtained after six passages (ca 20 population doublings). In the latter case, the control cells and cells exposed to 10^{-8} *M* PDD were also seeded in methylcellulose containing 10^{-7} *M* PDD to verify the importance of prior subculturing in PDD to obtain significant anchorage independent growth. The average colonies per plate for the control cells increased only from 5.5 to 21 under these conditions, whereas the average for the PDD treated cells increased from 7.5 to 83.5. In all cases, the few cell colonies obtained without added promoter were much smaller than those with promoter.

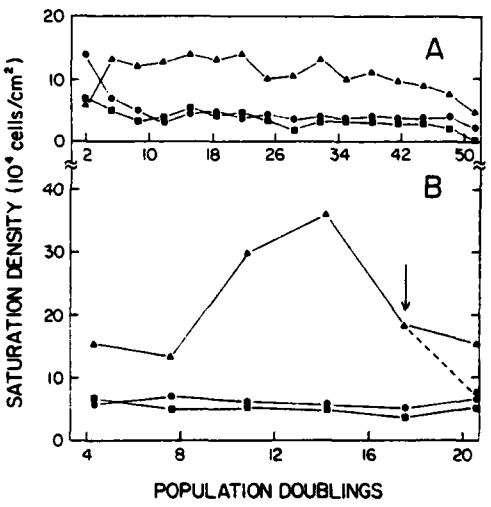

FIG. 1. Saturation densities of human diploid cells exposed continuously to phorbol esters. The cell populations are: Control (●), 10^{-7} *M* PDD (▲), and 10^{-7} *M* 4α-PDD (■). *A*. Human skin cells cultured in medium supplemented with 8X nonessential amino acids. The primary culture was split at a ratio of 1:4 after which the treated and untreated populations were subcultured at a ratio of 1:10 at 7 d intervals. The ordinate indicates control population doublings 2 to 51.5. *B*. Human skin cells cultured in medium supplemented with 1X nonessential amino acids plus 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine. Cell populations were subcultured at a ratio of 1:10, were fed with fresh medium after 3 d, and were enumerated after 7 to 8 d. The ordinate indicates control population doublings 4.3 to 20.8. The *arrow* denotes the subculture at which PDD was removed from duplicate cell populations (△; *broken line*), and also when additional postconfluent cultures were stained and photographed (Fig. 2).

DISCUSSION

The induction of transformed phenotypes in cultured normal human cells by phorbol ester tumor promoters has not been described although such promoter-induced mimicry of transformation is well known in cultured rodent cells (8). The data presented in this report demonstrate that, under appropriate culture conditions, tumor pro-

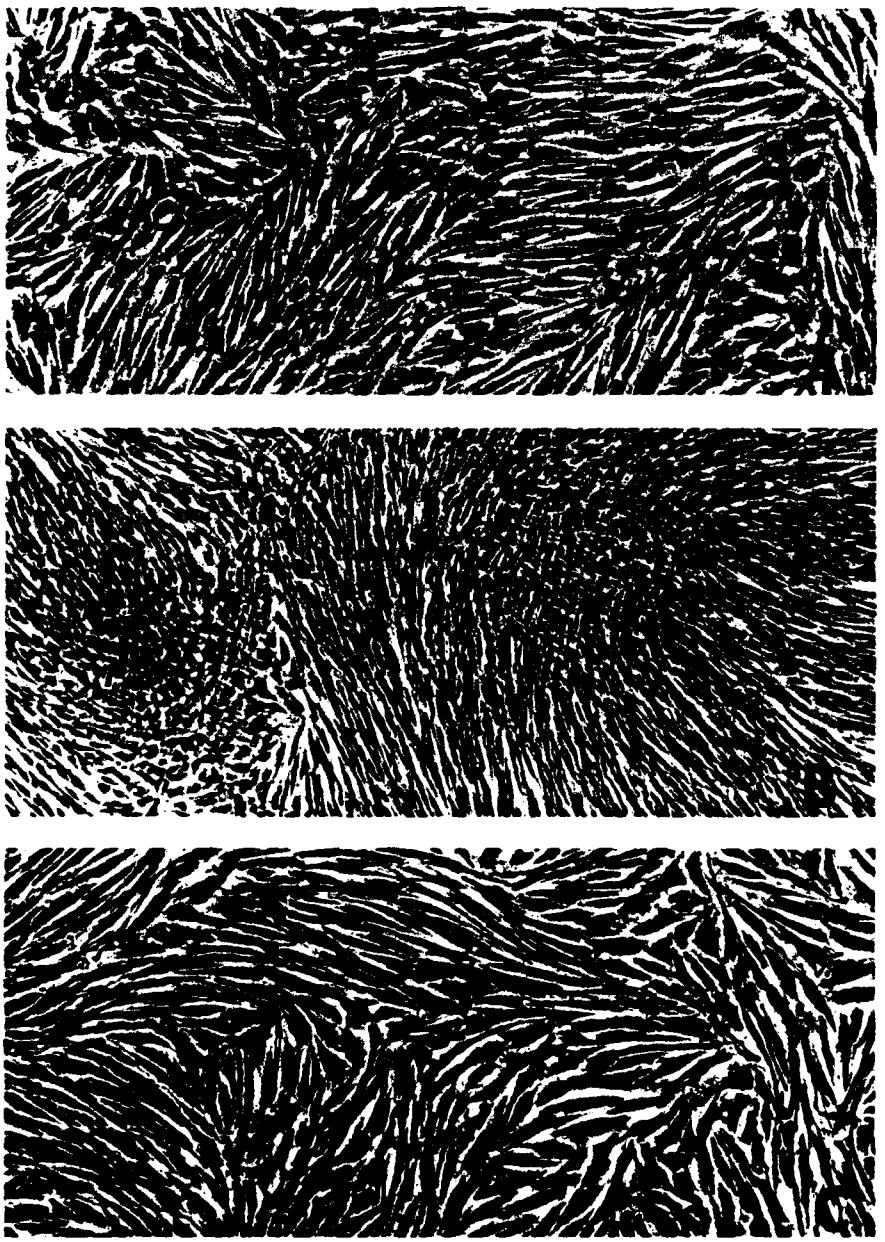

FIG. 2. Photomicrographs of control and phorbol ester treated human cells. Postconfluent cultures (Fig. 1 *B*, control population doubling level 17.5) were fixed in methanol 9 d after subculture and were stained with May-Grünwald Giemsa. *A*, Control; *B*, PDD treated; and *C*, 4α-PDD treated. ×220.

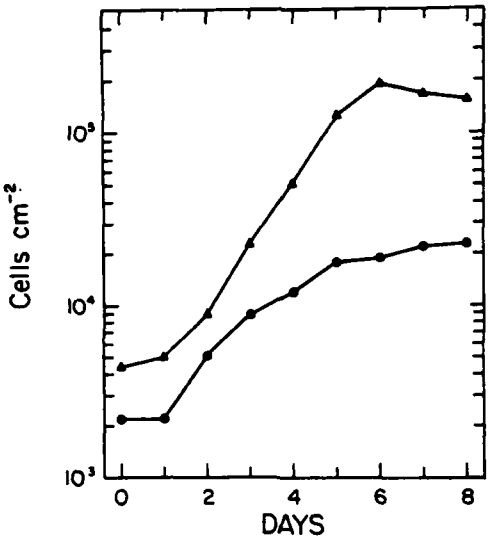

FIG. 3. Growth curves for human diploid cells grown in the absence and presence of PDD. The cell populations are: Control (●) and 10^{-8} *M* PDD (▲). Secondary postconfluent cultures (cultures having been exposed to 0.01% acetone ± 10^{-8} *M* PDD for 1 passage) in medium supplemented with 2X aspartic acid, asparagine, histidine, phenylalanine, and tyrosine were subcultured 1:10 (control) and 1:30 (PDD) on Day 0. Duplicate cultures were trypsinized and counted with a hemocytometer each day.

moters can also induce the expression of transformed phenotypes in early passage human skin cells. The loss of density dependent growth inhibition in monolayer culture as well as the anchorage independent growth induced by the phorbol ester PDD are characteristic transformed phenotypes expressed by human skin cells during neoplastic transformation induced by chemical and physical carcinogens in vitro (15). Therefore, PDD elicits a mimicry response in the absence of carcinogen initiation.

The increase in cell density induced by chronic exposure of human cells to PDD was appreciable (Fig. 1). Although it was suggested that smaller cell size (<30%) might explain the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced increases in saturation density (<50%) reported by Diamond et al. (9), it obviously could not explain the PDD-induced increases of up to 1000% in the present investigation. Although similar decreases in cell size were obtained under some conditions with PDD, the two- to tenfold increase in saturation density induced by PDD was not due to significantly more cells per unit area on the surface of the flask. As with carcinogen transformed human cells (15), the PDD treated cells were no longer sensitive to density dependent growth inhibition (Fig. 2 *B*). This is in contrast to the reported phorbol ester-induced inhibition of cell proliferation for sparse cultures of normal human cells under standard culture conditions (10). However, as with most studies involving cultured human cells, the phorbol ester utilized previously was TPA and the treatment period was short term. TPA has been reported both to inhibit and to stimulate proliferation of mouse fibroblasts depending on the culture conditions and timing employed (18). PDD was also somewhat inhibitory during the first exposure to the human cells in this study, and although quantitative comparisons were not undertaken, PDD always gave consistent increases in saturation density whereas TPA did not (data not presented).

Anchorage independent growth is not a common tumor promoter-induced phenomenon for normal rodent cells or normal human cells under standard culture conditions (3,4,12,19), i.e. it is not a common characteristic of mimicry of transformation for normal cells (8). However,

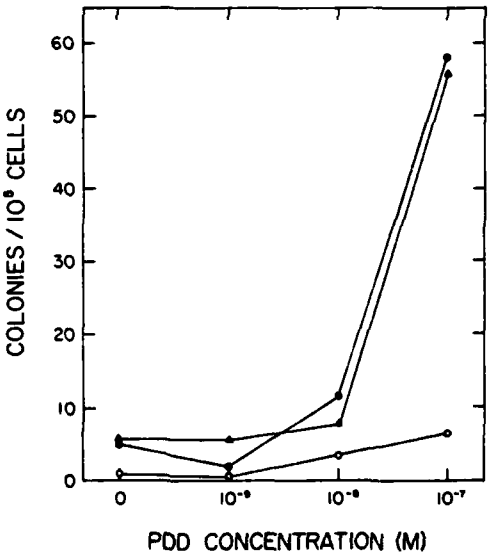

FIG. 4. Dose dependence of PDD-induced anchorage independent growth of normal human cells. The cells were seeded in methylcellulose after 2 passages (○), 4 passages (●), and 6 passages (▲) in 0, 10^{-9}, 10^{-8}, or 10^{-7} *M* PDD. Conditions as described in Fig. 1 *B* for growth and subculturing of cells and as in Materials and Methods for assessing anchorage independent growth. The methylcellulose overlay medium was supplemented with the indicated concentrations of PDD.

some carcinogen-initiated mouse epidermal cell lines and adenovirus-transformed rat embryo fibroblasts do exhibit increased anchorage independent growth in the presence of phorbol ester and nonphorbol ester tumor promoters, and the response occurs in a dose dependent manner (3,4,20). Normal human skin cells are also subject to phorbol ester-induced anchorage independent growth under the prescribed culture conditions (Fig. 4). This response is also dose dependent, but whereas the rodent cells only require short-term exposure to the phorbol ester, the human cells require long-term exposure (Fig. 4). Considering that repeated applications of a phorbol ester are necessary for tumor promotion on mouse skin after carcinogen initiation (1), the induction of anchorage independent growth by chronic exposure of normal human cells to PDD in vitro may be analogous to mimicry of tumor formation *in vivo*.

Further changes in the cell culture environment may make it possible to obtain even greater PDD-induced alterations in the growth properties of human diploid cells. However, variations in amino acid concentrations in the culture medium seem to be important to obtain reproducible effects from one primary culture to another. In addition, starting the phorbol ester treatment at the first passage of the primary culture enhances the observable cellular responses significantly. The in vitro transformation protocol of Milo et al. (15) utilizing the same cell type, requires that the cells undergo only a few population doublings before carcinogen treatment to attain neoplastic transformation. Therefore, human cell populations may become refractory to initiation and promotion of carcinogenesis with prolonged culture, i.e. they are modulated by the tissue culture environment. The different treatment protocol utilized might thus explain the significantly greater phenotypic response to a phorbol ester tumor promoter than demonstrated previously for normal human cells. Using this new protocol, it may be possible to study promotion of carcinogenesis after chemical carcinogen initiation using normal human cells in vitro.

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Relationship between a Tumor Promoter-induced Decrease in Queuine Modification of Transfer RNA in Normal Human Cells and the Expression of an Altered Cell Phenotype[1]

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ABSTRACT

With normal human skin fibroblasts in culture, a transient decrease in queuine modification of tRNA precedes a phorbol ester tumor promoter-induced 5- to 10-fold increase in saturation density. Subsequently, an increase in the queuine content of cellular tRNA (to levels comparable to those in untreated cultures) precedes a decrease in saturation density. This reversal of the phorbol ester-induced alteration in tRNA modification occurs in the continued presence of the tumor promoter, and it parallels an increased ability of the cells to salvage queuine from catabolized endogenous tRNA. Addition of exogenous queuine concurrently with the tumor promoter at early passage significantly inhibits the increase in saturation density. The results suggest a role for the decrease in queuine modification of tRNA in mediating the phenotypic change induced by the tumor promoter.

INTRODUCTION

Following synthesis of the primary transcript of individual tRNA species, the macromolecules are subjected to extensive modification by diverse, highly specific enzymes (1). One of the most unusual modification reactions is catalyzed by the enzyme tRNA-guanine ribosyltransferase (EC 2.4.2.29), which is involved in generating queuine[4]-containing tRNA (Chart 1). This modification occurs by a direct base replacement of queuine for guanine only in the first (wobble) position of the anticodon of tRNA isoacceptors for aspartic acid, asparagine, histidine, and tyrosine (11, 12, 18).

Mammalian cells obtain queuine from the diet or gut flora *in vivo* (3, 16) and from sera utilized to supplement cell culture media *in vitro* (7, 11). Although queuine is a dietary factor that is not synthesized by mammalian cells (3, 16), normal mature tRNAs are usually fully queuine modified; *i.e.*, they are in the $tRNA_Q$ form depicted in Chart 1. However, when mammalian cells undergo neoplastic transformation *in vivo* or *in vitro*, one of the most common tRNA structural changes involves a decrease in queuine modification (15). Under these circumstances, one or more of the isoacceptors for aspartic acid, asparagine, histidine, and/or tyrosine may have guanine instead of queuine in the anticodon wobble base position; *i.e.*, they are in the $tRNA_G$ form depicted in Chart 1. This decrease in queuine-containing tRNA in transformed cells has been postulated to arise from substrate limitation (10, 18) or enzyme inhibition (2, 9, 10, 18), but the functional significance of the altered queuine content in the tRNA anticodon has not been established. However, undermodification for queuine has also been reported in regenerating rat liver (15); therefore, such changes may not be associated directly with neoplastic transformation.

During a study of the chronic effects of phorbol ester tumor promoters on normal human cells in culture, it was observed that long-term 2- to 4-fold increases in saturation densities were obtained reproducibly only if the culture medium had been supplemented with excess amino acids (21). In addition, if these excess amino acids included aspartic acid, asparagine, histidine, and tyrosine, a transient 5- to 10-fold increase in the saturation density of early-passage cells also was induced by the phorbol esters (21). This large, transient increase in saturation density was not obtained with later-passage human cells, and phorbol esters which are inactive as tumor promoters on mouse skin did not affect the saturation density of the normal human cells *in vitro*. Because the amino acids utilized were those accommodated by queuine-containing tRNAs, the possibility that altered queuine modification of tRNA plays some role in the phorbol ester-induced increase in saturation density was investigated.

MATERIALS AND METHODS

Cell Culture. Primary cultures of normal human cells were prepared from neonatal foreskin as described by Riegner *et al.* (17), which results in a predominantly fibroblastic culture. At the first passage of the primary culture, the fetal bovine serum content in Eagle's MEM was reduced from 20 to 10%, and it was maintained at that level for subsequent passages. The MEM was also supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.2); 2 mM glutamine; 1 mM sodium pyruvate; gentamicin (50 μg/ml); 0.2% sodium bicarbonate; 2× vitamins; 1× nonessential amino acids; and 2× aspartic acid, asparagine, histidine, phenylalanine, and tyrosine (21). Cells were subcultured in 25-sq cm flasks at a ratio of 1:10 at 7- to 8-day intervals with medium replacement after the first 3 days. PDD and 4α-PDD (dissolved in acetone) were added to matched cultures at the first or second passage of the primary culture, and they were included with every subsequent subculture and feeding. Postconfluent cell populations (in duplicate) were trypsinized, and cells were counted with a hemacytometer to establish saturation densities.

For the queuine supplementation experiments, the primary cultures were established as described above but with 20% bovine serum replacing the fetal bovine serum. At the first passage of the primary culture, the bovine serum concentration in the medium was reduced to 10%, and the PDD and/or queuine were added. Queuine was purified from bovine

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[4] The abbreviations used are: queuine, 7-[[5-[[(1*S*,4*S*,5*R*)-4,5-dihydroxy-2-cyclopenten-1-yl]amino]methyl]]-7-deazaguanine (13); $tRNA_Q$, queuine-plus (Q^+) tRNA; $tRNA_{Q^-}$, queuine-minus (Q^-) tRNA; $tRNA^{His}$, histidine tRNA; $tRNA^{Asp}$, aspartic acid tRNA; PDD, phorbol 12,13-didecanoate; 4α-PDD; 4α-phorbol-12,13-didecanoate; MEM, minimal essential medium.

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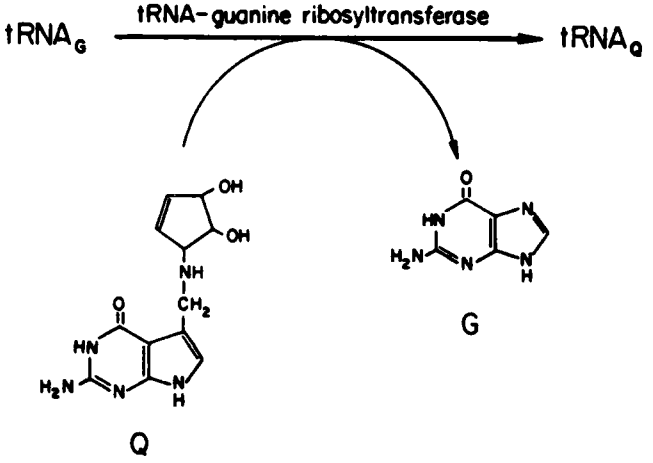

Chart 1. tRNA-guanine ribosyltransferase reaction. The enzyme exchanges queuine for guanine in the first position of the anticodon of mammalian tRNAs for aspartic acid, asparagine, histidine, and tyrosine. *Q*, queuine; *G*, guanine.

amniotic fluid (7, 10). Queuine was added to the medium at 3×10^{-8}, 5×10^{-8}, or 10^{-7} M, while PDD was maintained at 10^{-8} M. All other conditions were as described above, except that the cells were grown in 2-sq cm 4-well multidishes to conserve queuine. Postconfluent cell cultures (in duplicate) were trypsinized, and cells were counted with a hemacytometer.

Guanine Incorporation Assay. Proliferating cells in 150-sq cm flasks were harvested and homogenized in hypotonic buffer (24). Transfer RNA, isolated by phenol extraction and DEAE-cellulose column chromatography (11), was utilized as a substrate in the guanine incorporation assay of Okada *et al.* (15), as modified by Elliott and Trewyn (2). The assay involves the enzymatic exchange of radiolabeled guanine for the guanine in the first position of the anticodon of queuine-unmodified tRNAs ($tRNA_G$ in Chart 1) by the tRNA-guanine ribosyltransferase isolated from *Escherichia coli*; an enzyme not able to utilize the queuine-modified forms ($tRNA_Q$ in Chart 1) as a substrate. Therefore, radiolabeled guanine incorporation into tRNA is a measure of queuine hypomodification.

Transfer RNA-guanine ribosyltransferase was isolated from *E. coli* MRE600 cells as described by Okada and Nishimura (14). However, purification of the enzyme was carried only through the DEAE-cellulose chromatography step, since the preparation was free of RNase activity when the RNase-deficient strain MRE600 was utilized (2). The assay reaction mixture was a modification of that described by Howes and Farkas (6), which contained 10 μmol of Tris-HCl (pH 7.4), 53 μmol of KCl, 5 μmol of 2-mercaptoethanol, 1 nmol of [8-^{3}H]guanine (1 Ci/mmol), 0.05 to 0.25 A_{260} units of tRNA, and 6 units of *E. coli* tRNA-guanine ribosyltransferase in a total volume of 0.6 ml (2). After a 60-min reaction time at 37°, radiolabeled tRNA in triplicate assays was precipitated by adding 0.2 ml of ice-cold 30% trichloroacetic acid. The tRNA was collected on glass fiber filters, and was washed extensively with ice-cold 5% trichloroacetic followed by 95% ethanol prior to scintillation counting.

RPC-5 Chromatography. Purified tRNA from both PDD-treated and untreated human skin cells was aminoacylated with radiolabeled histidine using a mouse liver-derived aminoacyl-tRNA synthetase preparation. The histidyl-tRNA isoacceptors were separated on a column (0.9 x 20 cm) of RPC-5 support developed at 27° with a 150 ml of 0.475 to 0.80 M NaCl linear gradient in 10 mM sodium acetate buffer (pH 4.5), 10 mM $MgCl_2$, 3 mM 2-mercaptoethanol, and 1 mM EDTA; the flow rate was 1.4 ml/min, 1.5 ml fractions were collected, and the radioactivity was measured as described (7). Periodate oxidation of aminoacylated $tRNA^{His}$ prior to RPC-5 separation was performed as described previously (3, 9, 23). RPC-5 chromatographic analyses of $tRNA^{Asp}$ isoacceptors were also carried out as described previously (7).

Queuine Salvage. The primary culture was established as described above but with 20% bovine serum replacing the fetal bovine serum. Thereafter, the medium was supplemented with 10% bovine serum that had been treated with dextran-coated charcoal to remove queuine (7). To monitor the decay of [^{3}H]dihydroqueuine-tRNA in the human cells, cells in 60-mm plates were exposed for 24 hr to 2 ml of medium containing 1 μM [^{3}H]dihydroqueuine (0.12 Ci/mmol) as described by Gündüz and Katze (4, 5). The medium containing excess radiolabel was decanted, and the cell monolayers were rinsed with the medium described above. Further culture was performed in 5 ml of queuine-deficient complete medium or matched medium supplemented with 1.5×10^{-8} M queuine. At daily intervals, the medium was removed from duplicate cultures, the cell monolayers were washed with medium, and the cells were removed by scraping in 5% ice-cold trichloroacetic acid. The cells were transferred to glass fiber filters and were washed with ice-cold 5% trichloroacetic acid followed by 95% ethanol. The filter-bound, acid-insoluble radioactivity was determined by liquid scintillation. The acid-insoluble radioactivity reflects the amount of [^{3}H]dihydroqueuine retained covalently in the tRNA of the cell, and its half-life is related to the ability of the cell to salvage [^{3}H]dihydroqueuine from tRNA turnover in the absence of exogenous queuine (4, 5).

Materials. Neonatal foreskin was supplied by the Tumor Procurement Service of the Ohio State University Comprehensive Cancer Center. Fetal bovine and bovine serum were purchased from Sterile Systems, Logan, UT, and bovine amniotic fluid was from Irvine Scientific, Santa Ana, CA. Eagle's MEM and the medium supplements were purchased from Grand Island Biological Co., Grand Island, NY, while *E. coli* MRE600 cells were obtained from Grain Processing Corp., Muscatine, IA. The phorbol esters PDD and 4α-PDD were obtained from P-L Biochemicals, Milwaukee, WI, and Sigma Chemical Co., St. Louis, MO. [8-^{3}H]Guanine, [^{3}H]histidine, and [^{14}C]histidine were purchased from Amersham/Searle Corp., Arlington Heights, IL, and [^{3}H]dihydroqueuine (reduced queuine) was prepared from queuine by catalytic reduction and exchange (12), also performed by Amersham.

RESULTS

Significant decreases in the queuine content of tRNA (as measured by increased guanine incorporation) were observed in human cells treated continuously with 10^{-7} M PDD, and this change in queuine modification preceded the transient increase in saturation density promoted by PDD (Chart 2). The timing of the decrease in queuine content and increase in saturation density varied somewhat from one primary culture to another,

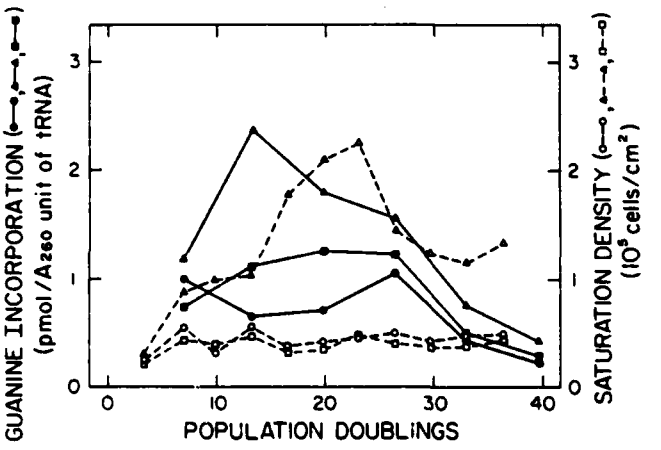

Chart 2. Decreased queuine modification of tRNA in human cells induced by PDD. The tRNA from control cells (●) and cells treated with 10^{-7} M PDD (▲) or 4α-PDD (■) was assayed for increased guanine incorporation (decreased queuine modification) at increasing cell population doublings in culture. The saturation densities of the control culture (○) and the PDD- (△) and 4α-PDD- (□) treated cultures are also indicated. See "Materials and Methods" for details.

but in 5 independent experiments, the maximal decrease in queuine modification preceded the maximal increase in saturation density as in the experiment depicted (Chart 2). In all experiments, an increase in queuine modification then preceded a decrease in saturation density. The structural analogue 4α-PDD, which is inactive as a tumor promoter on mouse skin, had a minimal effect on queuine modification and saturation density.

RPC-5 chromatographic analyses of the histidine tRNA isoacceptors from PDD-treated and untreated human cells demonstrated significantly different elution profiles (Chart 3). Control cell histidine tRNA species were predominantly in the queuine-modified (Q^+) form rather than the queuine-unmodified (Q^-) form (Chart 3*A*), *i.e.*, $tRNA_Q$ *versus* $tRNA_G$. However, cells treated continuously with 10^{-8} M PDD yielded tRNA with a majority of the histidine isoacceptors unmodified for queuine (Chart 3*B*). The identity of the queuine-modified isoacceptor was determined by periodate oxidation, which specifically (because of the cyclopentenediol moiety in queuine) retards the RPC-5 elution of queuine-containing tRNAs (23) (data not shown).

Based on the known molecular mechanism for queuine insertion into tRNA (Chart 1), the transient nature of the hypomodification induced in the continuous presence of PDD was unexpected. However, it was discovered that newly established cultures of human skin cells lack the ability to salvage queuine subsequent to tRNA turnover, whereas later passage cells acquire this ability (Chart 4). When first-passage human skin cells were radiolabeled with [^{3}H]dihydroqueuine (Chart 4*A*), the presence or absence of exogenous queuine had no effect on the half-life of the acid insoluble radioactivity, thereby indicating the lack of queuine salvage in these cells. However, at the fourth passage of cells derived from the same primary culture (Chart 4*B*), the half-life of radiolabeled tRNA in queuine-free medium was appreciably longer (>20 days) than that in queuine-containing medium (1.5 days), indicating the induction of the queuine salvage pathway. Again, the timing of induction of maximal queuine salvage from endogenous tRNA turnover varied somewhat from one primary culture to another, but it usually was attained by 15 to 20 population doublings (4 to 6 passages) in culture.

The serum utilized to supplement the cell culture medium contains queuine (medium supplemented with 10% fetal bovine serum contains 1 to 2 × 10^{-8} M queuine) (8), and this is the source of queuine for tRNA modification in cultured cells (7, 11). Since the above data suggested a role for queuine limitation in the PDD-induced increase in saturation density, the effect of queuine supplementation on the saturation density of human cells was examined (Chart 5). When compared to control cells in this experiment, 10^{-8} M PDD effected a maximum increase in

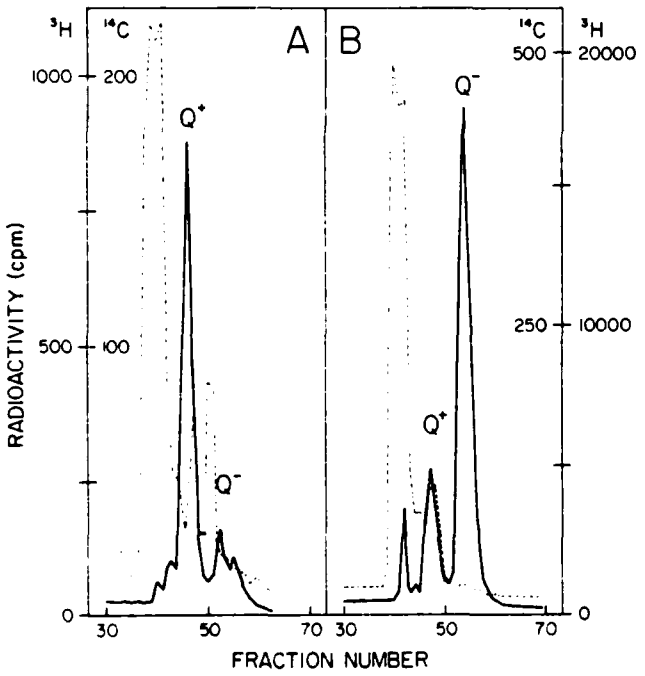

Chart 3. RPC-5 chromatographic profiles of $tRNA^{His}$. The *solid lines* represent (*A*) [^{14}C]histidine-tRNA derived from second passage human skin cells and (*B*) [^{14}C]histidine-tRNA derived from second passage human skin cells treated with 10^{-8} M PDD. Major human fibroblast elution *peaks* are designated the Q^+ ($tRNA_Q$) and Q^- ($tRNA_G$) isoacceptors, determined by periodate oxidation and RPC-5 chromotography (see text). *Dotted lines* represent [^{3}H]histidine-tRNA derived from normal mouse liver. Mouse liver $tRNA^{His}$ *Peak 1* (Fractions 35 to 45) and *Peak 2* (Fractions 45 to 49) have been shown to be Q^+ and Q^-, respectively (10). We postulate the similar elution properties of the Q^+ human fibroblast $tRNA^{His}$ and the Q^- mouse liver $tRNA^{His}$ to result from a second elution-altering modification difference between the 2 tRNA species. This will be described further elsewhere. The mouse liver $tRNA^{His}$ peak which elutes between Fractions 49 to 52 in *A* and is absent in *B* is an artifact which developed with prolonged use of a single aqueous solution of mouse liver tRNA. See "Materials and Methods" for details.

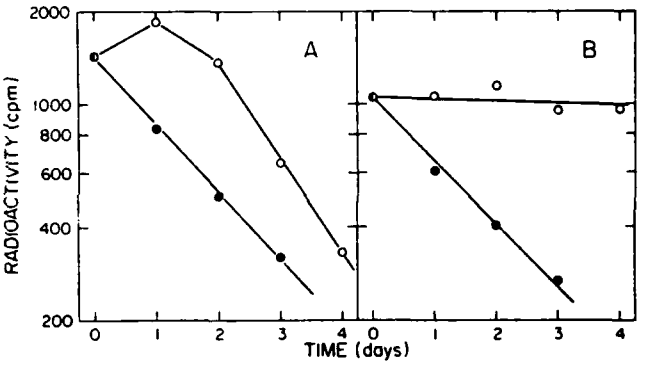

Chart 4. The decay of [^{3}H]dihydroqueuine-tRNA in normal human cells. The same primary culture was subpassaged at a ratio of 1:10 one (*A*) and 4 (*B*) times. The results for the cells in queuine-deficient medium (○) and medium supplemented with 1.5 × 10^{-8} M queuine (●) are indicated. The mean of duplicate determinations is presented. See "Materials and Methods" for details.

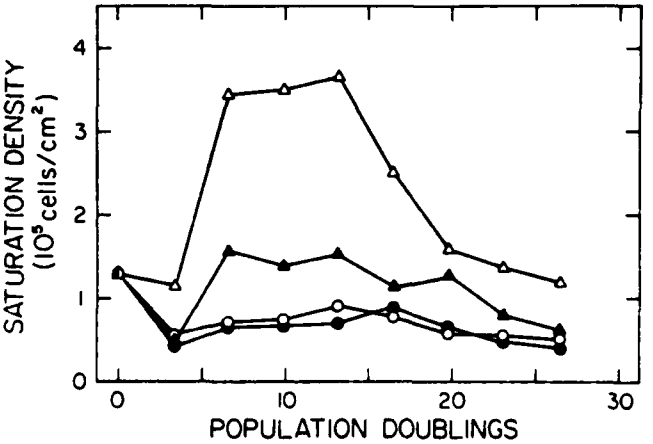

Chart 5. Saturation densities of human cells exposed continuously to PDD with or without excess queuine. The cell populations are: ○, control; ●, control plus 5 × 10^{-8} M queuine; △, 10^{-8} M PDD; and ▲, 10^{-8} M PDD plus 5 × 10^{-8} M queuine. See "Materials and Methods" for details.

saturation density of approximately 5-fold without added queuine but only 2-fold with pure queuine added at 5×10^{-8} M. In separate experiments (not shown), adding 10^{-7} M queuine was no more effective (than 5×10^{-8} M queuine) in suppressing the PDD-induced increase in saturation density, while 3×10^{-8} M queuine was slightly less effective. The saturation densities of control cells were not altered significantly by 5×10^{-8} M queuine (Chart 5), but they were decreased slightly by 10^{-7} M (not shown).

DISCUSSION

Phorbol ester tumor promoters elicit numerous changes in the growth characteristics of mammalian cells *in vitro* (22). However, there have been few reports of these agents significantly altering the growth properties of normal human cells. The recent finding that early-passage human cells are also affected by the phorbol esters if the culture medium is supplemented with elevated levels of the amino acids accommodated by queuine-containing tRNAs (21) led to the present investigation.

Studies were undertaken to analyze the queuine content of tRNA isolated from human skin cells grown in culture medium with or without PDD. Changes in queuine modification of cellular tRNAs were compared to the saturation density changes induced by the phorbol ester. In those cells treated continuously with 10^{-7} or 10^{-8} M PDD, a significant transient decrease in the queuine content of tRNA was observed, and this was followed by a substantial, but transient, increase in saturation density (Chart 2). With further passage of the PDD-treated cultures, the queuine content of the tRNA increased to that of control cells, while the saturation density decreased toward that of control cells. However, the saturation density of the PDD-treated cells routinely remained 2 to 3 times that of control following the transient 5- to 10-fold increase.

RPC-5 chromatography of histidine tRNA isoacceptors from the PDD-treated and untreated human skin cells demonstrated that the histidine tRNAs from these cells were significantly different (Chart 3). Exposure of the cells to PDD induced a decrease in the modification of the histidine tRNA isoacceptors of the cell with respect to queuine, and this result correlated to the induction of queuine hypomodification in the unfractionated tRNA observed in Chart 2. RPC-5 chromatography of $tRNA^{Asp}$ isoacceptors from the PDD-treated and untreated cells demonstrated no significant differences (data not shown). However, aspartic acid tRNAs have been shown to be as much as 5-fold better substrates for queuine incorporation than are the other 3 potential queuine-containing tRNA species (10) so, under conditions of queuine limitation (such as those apparently induced by PDD), histidine tRNAs should become queuine-hypomodified before aspartic acid tRNAs.

The necessity for early-passage human cells to obtain the major PDD-induced increase in saturation density (21) appears to be explained by the induction of queuine salvage with time in culture. Salvage allows the reuse of queuine derived from tRNA turnover (4, 5), thereby decreasing the exogenous queuine requirement of the cells. We have used a simple intact cell assay (4) to test for queuine salvage activity. Cells are cultured first in the presence of [^{3}H]dihydroqueuine to label the tRNA, then rinsed and cultured further in unlabeled medium which is either queuine-free or queuine-supplemented, and finally, the label from [^{3}H]-dihydroqueuine-tRNA is followed with time. In the presence of exogenous queuine, the decay of label is equal to that of tRNA turnover, while a relatively diminished decay in the absence of queuine indicates the [^{3}H]dihydroqueuine is salvaged after tRNA turnover and that it is reattached to newly synthesized tRNA (4). Minimal salvage is observed in very early-passage cells (Chart 4*A*), while significant salvage is indicated in later-passage cells (Chart 4*B*). As a result of the induction of the queuine salvage pathway with time (Chart 4) or the addition of queuine to the culture medium (Chart 5), PDD would be less able to inhibit queuine modification of tRNA, which appears to be required to obtain the greatest increase in saturation density.

While previous studies have not elucidated a specific role for the decreased queuine content of tRNA in carcinogenesis, work by Katze and Beck (9) demonstrated that the administration of queuine to tumor-bearing mice reversed the queuine hypomodification of tRNA and apparently inhibited tumor growth as well. Elliott and Trewyn (2) also reported a correlation between a 7-methylguanine-induced decrease in queuine modification of tRNA and transformation of Chinese hamster embryo cells *in vitro*, and Trewyn *et al.* (20) postulated a role and model for this methylated purine-induced queuine hypomodification in the expression of carcinogenesis. In addition, studies by Shindo-Okada *et al.* (19) with murine erythroleukemia cells *in vitro* indicated a relationship between queuine modification of tRNA and cellular differentiation induced by dimethyl sulfoxide, both processes being inhibited by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate.

The present data offer a direct indication that queuine hypomodification of tRNA in human skin cells is correlated to a tumor promoter-induced phenotypic change, *i.e.*, an alteration in saturation density. These results suggest that the decrease in queuine content in the anticodon of tRNAs for aspartic acid, asparagine, histidine, and/or tyrosine observed in neoplasia could be involved in promotion of carcinogenesis. Based on the pivotal role of tRNA in translation of the genetic code, this could help explain the pleiotropic changes induced by tumor promoters *in vitro* and *in vivo*.

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AD-A150 962 CHEMICAL CARCINOGEN-INDUCED CHANGES IN TRNA METABOLISM IN HUMAN CELLS(U) OHIO STATE UNIV RESEARCH FOUNDATION COLUMBUS R W TREWYN 20 NOV 84 AFOSR-TR-85-0098 2/2

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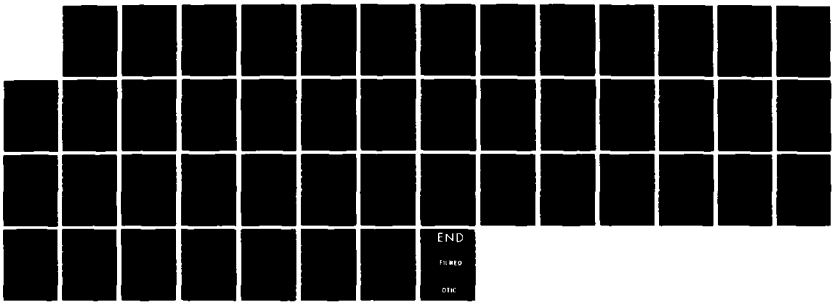

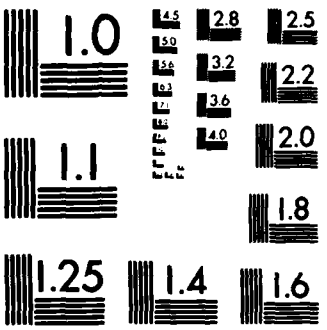

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Inhibition of Queuine Uptake in Cultured Human Fibroblasts by Phorbol 12,13-didecanoate[1].

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Running Title: Phorbol ester inhibition of queuine uptake

FOOTNOTES

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3. The abbreviations used are: queuosine or Q, 7-{5-[((1S,4S,5R)-4,5-dihydroxy-2-cyclopenten-1-yl)amino]methyl}-7-deazaguanosine (25), queuine is the corresponding base; rQT_3, queuine reduced with tritium in the cyclopentene ring and with H replaced by ^3H at C-8 (purine numbering system) in the deazaguanine nucleus; RPC-5 reversed-phase 5 chromatography; HPLC, high performance liquid chromatography; FBS, fetal bovine serum; PDD, phorbol 12,13-didecanoate; 4 α-PDD, 4α-phorbol 12,13-didecanoate; MEM, minimal essential medium; PMEM, MEM modified to optimize for phenotypic effects induced by phorbol ester tumor promoters (35).

ABSTRACT

The modified base queuine is inserted post-transcriptionally into the first position of the anticodon of $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$ and $tRNA^{Asp}$. Phorbol 12,13-didecanoate (PDD) effects a decrease in the queuine content of tRNA in cultured human foreskin fibroblasts. The present data suggest that this results from a PDD mediated inhibition of queuine uptake. Nonsaturable uptake was observed for tritiated dihydroqueuine (rQT_3) for up to 2 hours at 10-1000 nM concentrations, while saturation of uptake was observed after 3 to 4 hours. Lineweaver-Burke analysis of concentration vs uptake revealed biphasic uptake kinetics with high and low Km components of approximately 350 nM and 30 nM respectively. Competition by queuine of rQT_3 uptake indicated that both compounds have equal affinity for the uptake mechanism. PDD inhibited rQT_3 uptake, but required 30 to 60 minutes of exposure before the uptake was completely blocked. The rQT_3 efflux rate from cells was found to be 3 to 4 times greater than that of uptake, and PDD also inhibited the efflux reaction. The potential inhibitors furosemide, nitrobenzylthioinosine, ouabain, 7-methylguanine, 7-deazaguanine, guanine, guanosine, adenine, adenosine, hypoxanthine and epidermal growth factor had no effect on rQT_3 uptake. However, dipyridamole was immediately effective at reducing rQT_3 uptake.

Introduction

The modified nucleoside queuosine (Q)[3] is found exclusively in the first position of the anticodon for tRNAs accommodating the amino acids asparagine, aspartic acid, histidine and tyrosine (15,25). E. coli synthesizes Q-containing tRNA de novo, first exchanging 7-aminomethyl-7-deazaguanine into tRNA and subsequently modifying this to Q (28). Q in eukaryotic tRNAs arises as a post-transcriptional modification to these tRNAs by a base for base exchange of preformed queuine, the base of Q, for the guanine in the primary transcript (19,30). Mammals must obtain queuine from their diet or gut flora (10,33). Mammalian cells grown in culture obtain queuine from the animal sera used to supplement the culture media (16,19). However, tRNA isolated from neoplastically transformed cells often is significantly undermodified with respect to Q, i.e, these tRNAs contain guanosine in the position normally occupied by Q (18,25,29).

Several factors of potential significance to the control of queuine modification of tRNA in normal and neoplastic cells were discussed previously (18): dietary availability of queuine, transport rate, tRNA synthesis rate, insertion rate, possible competitor levels, catabolic rate, tRNA half-life and queuine salvage capability. The queuine insertion enzyme, tRNA-guanine ribosyltransferase (EC 2.4.2.29), has been reported to be present at roughly equivalent levels in both normal and neoplastic cells (25,30). 7-Methylguanine, an inducer of neoplastic transformation of Chinese hamster embryo cells in culture, is an inhibitor of tRNA-guanine ribosyltransferase in vitro and causes Q hypomodification of tRNA in intact cells (8). Naturally occuring pteridines also inhibit queuine incorporation into tRNA both in vitro and in vivo (11). Regarding salvage, the enzymatic activities enabling queuine retrieval subsequent to tRNA degradation have been identified (13,14); however, it remains to be determined if a deficiency in queuine salvage is an important cause of Q hypomodification in tumors.

In a preceding study involving phorbol ester tumor promoters and human fibroblasts in culture, we demonstrated that queuine modification of tRNA was inhibited by PDD (7). The decrease in the Q content in tRNA always preceded an increase in cell saturation density, and subsequently (with time in culture), an increase in the queuine content of tRNA (to levels comparable to those in untreated cultures) preceded a decrease in saturation density. The reversal of the PDD-induced alteration in tRNA modification occurred in the continued presence of the tumor promoter, and it paralleled an increased ability of the cells to salvage queuine from catabolized endogenous tRNA. Moreover, the addition of exogenous queuine concurrently with the PDD significantly inhibited the PDD-induced increase in saturation density. Thus, the concentration of queuine and the Q content of tRNA were inversely related to the PDD-effected increase in saturation density. Similarly, during erythroid differentiation in Friend erythroleukemia cells, a significant increase in the Q-content of cellular tRNA was reported to precede any detectable increase in hemoglobin content; both differentiation, as measured by hemoglobin content, and the increased Q-content of tRNA were effectively blocked by the tumor promoter 12-O-tetradecanoyl-phorbol 13-acetate (31). However, PDD does not inhibit the base exchange activity of tRNA-guanine ribosyltransferase (unpublished results). Therefore, because phorbol esters interact predominantly with membranes and membrane associated proteins (3,26), we considered the possibility that phorbol esters may inhibit queuine transport into the cell. The present study was undertaken to identify the functional characteristics of queuine uptake, as well as potential inhibitors of this process; e.g. phorbol esters, methylated purines, and purine analogs.

Materials and Methods

Materials: Fetal bovine serum was obtained from Dutchland (Denver, PA). PDD and its inactive analog 4 α-PDD were obtained from Sigma (St. Louis, MO.).

Queuine was isolated from bovine amniotic fluid (Irvine Scientific, Santa Anna, CA.) as previously described (20). rQT_3 (3.0 Ci/mmole) was prepared from queuine by catalytic reduction and exchange (20) by New England Nuclear (Boston, MA). Nitrobenzylthioinosine was a gift of Dr. Judith Belt. Epidermal growth factor was from Collaborative Research (Waltham, MA).

Cell Culture: Monolayer cell cultures were maintained in minimal essential medium (MEM) (GIBCO, Grand island, NY.) modified to optimize for phenotypic effects induced by phorbol ester tumor promoters as previously described (35). The MEM was supplemented with 25 mM HEPES (pH 7.2), 1.0 mM sodium pyruvate, 1x non-essential amino acids, (Gibco) 2x vitamins (Gibco), 0.1 mM asparagine (2x non-essential), 0.1 mM aspartic acid (2x non-essential), 0.2 mM histidine (2x MEM), 0.2 mM phenylalanine (2x MEM), and 0.2 mM tyrosine (2x MEM). This modified MEM is designated PMEM. The other supplements were obtained from Sigma. All medium was further supplemented with an additional 0.2% sodium bicarbonate, penicillin (100 units/ml) and streptomycin (100 µg/ml), prior to use, and the glutamine level was maintained at 2 mM.

Primary human skin cell cultures were established from neonatal foreskin according to the procedure of Riegner et al. (34). Neonatal foreskins were collected from local hospitals and were stored at 4°C for no more than one week in MEM supplemented with 5% FBS. Tissue was prepared for culture by mincing with crossed scapels and overnight exposure to 0.25% collagenase in PMEM supplemented with 20% FBS. The cell suspension was collected by centrifugation at 500 x g for 5 minutes. The cell pellet was resuspended in 10 ml of PMEM plus 20% FBS and seeded into a 75 cm^2 flask. The flask was incubated 24 hours at 37°C under a 4% CO_2 atmosphere, and then the medium was changed for fresh medium. When this primary culture reached confluence, the cells were considered to be at passage level zero. Subsequently, confluent monolayers were sub-cultured by rinsing the cell sheet with versene buffer, and treating with a 0.1%

trypsin solution in versene buffer. Trypsinization was halted by addition of PMEM with 10% FBS and cells were subcultured at a ratio of 1:4 into 150 cm^2 flasks or at a ratio of 1:2 into 35 mm dishes (10 cm^2 surface area). As these cells achieved confluence their media was changed to PMEM plus 10% queuine-depleted FBS, for 4 to 12 hours in order to deplete the cell's free queuine levels. Queuine depleted-FBS was generated by treatment of FBS with dextran coated charcoal (1,16). The confluent 35 mm dishes were rinsed once with serum-free medium and covered with 1.0 ml of serum-free medium which was unsupplemented (control) or with phorbol ester (100 nM PDD). These incubation media were further supplemented with $^{r}QT_3$ (10 nM to 1.0 μM), a radiolabeled reduced analog of queuine (20), which acted as the transport substrate. Progressive times of exposure from seconds to hours were performed at 37°C and termination of incubation was with three 3 ml rinses of the cell sheet with ice-cold phosphate buffered saline containing 10 μM dipyridamole (a general transport inhibitor) (36). The cells were lysed in 0.75 ml of 95% ethanol for 5 minutes, after which the lysate was aspirated and analyzed for radioactivity by liquid scintillation.

Efflux studies were performed on human skin cell cultures by incubating the cells in the presence of 1 ml of rQT_3 (1.0 μM) for 1 hour at 37°C. The cells were quickly rinsed twice with serum-free medium, and covered with 2 ml of the serumless media. Dishes were incubated for various additional times at 37°C. Incubations were terminated and cells were lysed as before.

HPLC: Human fibroblasts treated for one hour at 37°C with 0.50 μM rQT_3 were rinsed twice with ice-cold phosphate buffered saline containing 10 μM dipyridamole. The cells were treated with 60% methanol for 30 minutes, then scraped, transferred to tubes, which then were centrifuged (12,000 x g for 5 minutes), and the resultant supernatant was evaporated to dryness with a Savant Speed Vac Concentrator. The residue was dissolved in 150 μl of water and 50 μl samples were injected onto an HPLC column, (Alltech C18 5μ, 4.6 mm x 25 cm)

equilibrated and developed with 50 mM ammonium formate (pH 4.0) containing 5 % methanol, at a flow rate of 1 ml/minute. Fractions were collected and samples were analyzed for radiolabel by liquid scintillation and compared to standards.

Results

Chart 1

Chart 2

Chart 3

Chart 4

The concentration dependence of rQT_3 uptake by human skin cells is shown in Chart 1. Addition of queuine to the incubation media reduced rQT_3 uptake proportionate to the amount of added queuine, indicating similar affinities of the transport system for both queuine and its radiolabeled, reduced analog (Chart 2). The uptake appears to become biphasic at high concentrations. At rQT_3 concentrations similar to those of queuine in bovine and human sera (1-10 nM) (17), a Km of 30 $\pm$ 4 nM was determined for rQT_3 uptake (Chart 3). At higher rQT_3 concentrations, the Km of uptake was 350 $\pm$ 150 nM. While the high concentration uptake kinetics were somewhat variable from one primary culture to another, the Km of rQT_3 uptake in the physiological range was reproducible, and the distinct biphasic character was always observed (five independent experiments). Short term rQT_3 uptake identified a rapid uptake component that appeared to saturate within 2 to 4 minutes, followed by a slower uptake component (Chart 4). The slower component saturated after several hours incubation with rQT_3 at 37°C.. Uptake was not inhibited by 1 mM furosemide (an inhibitor of K^+ transport) (27), 10μ M nitrobenzylthioinosine (an inhibitor of nucleoside transport) (2), or 2 mM ouabain (an inhibitor of Na^+ -K^+ ATPase) (21). The purines guanine, adenine, hypoxanthine, 7-methylguanine, and 7-deazaguanine, along with the nucleosides guanosine and adenosine (concentrations of 10-20 μM), had no inhibitory effect on rQT_3 uptake (20 nM) (data not shown). However, treatment with 10 μM dipyridamole (a general transport inhibitor) (36) immediately reduced transport rates by 50 to 70 % (data not shown).

Treatment of cell cultures with 100 nM PDD resulted in inhibition of rQT_3 uptake but the onset of inhibition was delayed and occurred by 30 to 60 minutes at 1.0 μM rQT_3 (Chart 5). Similar results were obtained for reduced concentrations of rQT_3 (down to 100nm). At rQT_3 concentrations between 10 and 50nm the onset of PDD induced inhibition began to lengthen from 60 minutes to 90 minutes as rQT_3 concentrations decreased (data not shown). Exposure of cells to PDD prior to the addition of rQT_3 resulted in only a modest increase in the inhibition of rQT_3 uptake. Relative to control cells, the one hour uptake of rQT_3 was 69% when PDD and rQT_3 were added simultaneously, and 52% when PDD was added 6 hours prior to rQT_3 (data not shown). PDD exerted inhibitory effects on the low Km portion of the uptake mechanism (Km = 30 nM) while not appreciably altering the higher Km component of uptake (Chart 6). In agreement with our previous study (7), in which the maximum PDD-effected decrease in Q-containing tRNA was observed with early passage cells, the PDD-effected inhibition of queuine uptake was reliably observed only in early passage cells (passages 1 to 4, corresponding to 1 to 8 population doublings). The inhibition of rQT_3 uptake by PDD was not affected by concurrent treatment with 0.1 mM quercetin (Chart 7). Epidermal growth factor (100 nM) also had no affect on rQT_3 uptake (data not shown).

Chart 5

Chart 6

Chart 7

When cells which had been preloaded with 1μM rQT_3 were placed in unlabeled medium, complete efflux of "unbound" rQT_3 occurred within 15 min (Chart 8A). The presence of 100 nM PDD during the efflux period resulted in no inhibition; however, if 100 nM PDD were present during the one hour rQT_3 loading period as well as during efflux, rQT_3 efflux was almost completely blocked (Chart 8B). Efflux also was blocked by 10 μM dipyridamole, a component of the termination and rinse buffer.

Chart 8

Confluent cells in 100 mm dishes were incubated with 500 nM rQT_3 for one hour at 37°C, then lysed in methanol, centrifuged, and the supernatant was evaporated to dryness. The residue was analyzed by HPLC and the radiolabel was

found to be entirely in the position of rQT_3 with no nucleoside or nucleotide derivatives present (data not shown). This result rules out significant secondary metabolism of rQT_3 once it is transported into the cell, other than limited incorporation into tRNA by tRNA-guanine ribosyltransferase. Of the total amount of rQT_3 taken up by the cells, approximately 3% to 5% was incorporated into the tRNA by the end of the one hour incubation period.

Discussion

Measurement of rQT_3 uptake for periods varying from seconds to hours, demonstrated biphasic uptake rates. One rapidly saturable uptake (13.5 pmole/hr/10^6 cells) was followed at approximately 5 minutes by a second, slower uptake (2.3 pmole/hr/10^6 cells) which continued linearly for several hours until equilibrium was established. The slower uptake was saturable at 3 to 4 hours and extrapolated through the origin in zero time adjusted graphical analysis. Competition of rQT_3 uptake with unlabelled queuine demonstrated that the uptake mechanism has the same affinity for both compounds (Charts 2 and 5), thereby permiting the direct determination of queuine uptake characteristics using rQT_3. It is notable that tRNA-guanine ribosyltransferase exhibits a 10 to 50 fold preference for queuine over rQT_3 (11,18).

A variety of potential inhibitors were tested. Neither furosemide nor nitrobenzylthioinosine inhibited rQT_3 uptake, arguing that queuine transport does not occur through the common nucleoside transporter or through symport with K^+ (2,27). 7-Methylguanine, and 7-deazaguanine also were ineffective as inhibitors of rQT_3 uptake, even though both are inhibitors of tRNA-guanine ribosyltransferase insertion of queuine into tRNA (8,11,30). Ouabain, an inhibitor of Na^+ K^+-ATPase, did not affect rQT_3 uptake either, indicating that the process of rQT_3 uptake is energy independent. Epidermal growth factor, which activates

a tyrosine specific protein kinase (5,6), did not affect rQT_3 uptake. However, 10 μM dipyridamole effectively inhibits rQT_3 uptake and efflux in human fibroblasts. Therefore, dipyridamole was routinely included in the incubation termination buffer.

Treatment of human fibroblast cultures with 100 nM PDD inhibited long term uptake of rQT_3 into the cells, while the inactive tumor promoter 4 α-PDD (100 nM) did not. The time lag between phorbol exposure and the observed transport inhibition could be explained by the induction of secondary effectors. Therefore, we considered the possibility that phorbol ester activation of protein kinase C may mediate the inhibition of queuine transport. Phorbol esters have been demonstrated to interact specifically with and to activate protein kinase C (22-24); however, the question as to whether this is the sole phorbol receptor is unresolved (3,4). Therefore, we examined the affect on queuine uptake of quercetin, a flavanoid inhibitor of protein kinase C which binds to a site separate from the phorbol ester binding site (12). Quercetin did not relieve the PDD-effected inhibition of rQT_3 uptake. Although some residual activity of membrane bound protein kinase C may still be responsible for the delayed appearance of rQT_3 uptake inhibition, it is also possible that PDD interacts directly with a queuine transport component of the membrane.

Kinetic analyses of rQT_3 uptake across a broad concentration range revealed distinctly biphasic kinetics of uptake. At low queuine concentrations (similar to the physiological queuine concentration of various sera) the apparent Km of uptake was determined to be 30 $\pm$ 4 nM, with an associated Vm of 0.33 pmol/hr/10^6 cells. With higher, non-physiological rQT_3 concentrations, the apparent Km of uptake was approximately an order of magnitude greater (350 $\pm$ 150 nM), with an associated Vm of 22.5 $\pm$ 2.5 pmole/hr/10^6 cells. This variable high

concentration uptake may not be physiologically significant, but rather may represent nonspecific variation from culture to culture. Uptake in the physiological concentration range was shown to be inhibited by 100 nM PDD, whereas no effects were observed with the higher concentration range.

An important consideration in transport studies is metabolism of the substrate once it has passed through the cell membrane. The only known reaction that queuine undergoes within the cell is the enzymatic exchange for guanine in the first position of the anticodon of $tRNA^{Asp}$, $tRNA^{Asn}$, $tRNA^{His}$ and $tRNA^{Tyr}$ (14,20,25). Queuosine and its nucleotide are generated during tRNA turnover and queuine is regenerated from these as a result of a queuine salvage pathway (13,14). Human fibroblasts exposed for one hour in culture to 500 nM rQT_3 were lysed and studied by HPLC analysis. Very little rQT_3 was incorporated into cellular tRNA during this period (approximately 3% to 5% of total internal counts), primarily because the tRNA was already saturated with queuine prior to the uptake study. No metabolism of internalized rQT_3 to queuine derivatives was determined once it crossed the cell membrane. Therefore, uptake rates are due to transport and are not complicated by secondary metabolism.

Human fibroblast cultures preloaded with 1.0 μM rQT_3, and then placed in queuine-free medium, exported unbound rQT_3 from the cell monolayer almost completely within 15 to 20 minutes. This indicates a significantly greater rate (3 to 4 fold) of queuine efflux than influx. Some rQT_3 remained associated with the cells for 30 to 60 minutes, suggesting the presence of high affinity binding sites on the membrane and/or in the cellular cytosol. From the amount of rQT_3 retained after efflux from cells previously loaded with rQT_3 and the cell number, we estimated that there are approximately 3.5×10^5 apparent queuine binding sites per cell in the human fibroblast cultures. This number could include rQT_3 bound to tRNA, the transporter mechanism, tRNA-guanine ribosyltransferase or other proteins.

The early, saturable uptake component and the retention of rQT_3 after efflux argue for the existence of a high affinity queuine binding protein. Additional support for the participation of such a queuine binding protein in queuine transport comes from the low Km of uptake (which closely matches the Km of the ribosyltransferase responsible for queuine insertion into tRNA), and the rapidly saturated component of the uptake rate. At high queuine concentrations the binding protein would become saturated and this is consistent with the observed decrease in uptake kinetics specificity under such conditions, possibly reflecting a passive influx of queuine. Presumably, the queuine binding protein facilitates uptake by direct interaction on the inner surface of the cell membrane with the queuine transporter. In suppport of such a model, approximately 5% of the internalized queuine is associated with the membrane fraction of the cell (data not shown).

PDD may interfere with the passage of queuine from the membrane to the binding protein. In this regard, we note the recent suggestion that phorbol ester tumor promoters and queuine may share sufficient structural features to compete for the same cellular receptor(s) (32). However, the fact that the PDD-effected inhibition is evident only 30 to 60 minutes after queuine is added to the system, regardless of the length of time of prior exposure to phorbol ester, suggests that the action of PDD is indirect and that a queuine-dependent process also is involved. Saturation of a queuine binding site may be required before the phorbol ester can interact to eliminate further uptake.

We have reported previously on the correlation of a decreased Q content of tRNA and the phenotypic changes induced by PDD (7). The present data indicate that PDD decreases the Q content of tRNA by inhibiting queuine transport. PDD inhibition of the low Km but not the high Km uptake component, predicts that the phorbol ester inhibition of queuine uptake may be countered by exposure to elevated

amounts of queuine. This prediction is consistent with the observation that high levels of exogenous queuine can reverse a PDD induced increase in cell saturation density (7). The reversibility of phorbol induced effects by addition of excess queuine (7) suggests that a deficiency in queuine and/or queuine modified tRNAs may mediate tumor promotion, and, furthermore, that queuine may be an anti-promoting agent.

We wish to acknowledge, Dr. Judith A. Belt of St. Jude Children's Research Hospita for valuable consultation on techniques for initial rate studies, and Pat "Whooper" Moore for expert technical assistance.

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Chart 1. Time course of uptake for rQT_3 . Confluent cell monolayers (passage 10) in 35 mm dishes were covered with 1 ml of serumless media containing 10 nM (0), 20 nM (●), 30 nM (△),50 nM (▲) and 100 nM (■) rQT_3. Points represented are the mean of duplicate samples, following the conditions stated in Methods.

Chart 2. Inhibition of rQT_3 uptake by queuine. Confluent cell monolayers in 35mm dishes were covered with 1 ml of serumless media containing 100nM rQT_3. Dishes were divided into four groups including one control (△), and the others supplemented with queuine at final concentrations of 100nM (▲), 200nM (O) and 400nM (●). Points represent the mean of duplicate samples, following the conditions stated in Methods.

Chart 3. Lineweaver-Burk analysis for rQT_3 uptake. Low concentration region: Km = 30 $\pm$ 4 nM, Vm = 0.33 pmol/hour/10^6 cells. High concentration region Km = 350 $\pm$ 150 nM, Vm - 22.5 $\pm$ 2.5 pmol/hour/10^6 cells. This graph (derived from cells at passage 6) is representative of five independent studies (of cells from passages 1 to 9), in which kinetic constants were determined by linear regression and statistically analyzed to yield the above numbers. All data points were gathered in duplicate from confluent cell monolayers in 35 mm dishes covered with media containing 10-1000 nM rQT_3 and incubated for one hour. Termination of incubation was as outlined in Methods.

Chart 4. Time course of uptake for rQT_3 at early and late time points. Confluent cell monolayers (passage 6) in 35 mm dishes were covered with 1 ml of media supplemented with 100 nM rQT_3. The incubations were terminated at 10 second intervals in duplicate for the first minute of incubation at 37°C, then terminated at minute intervals from one minute to the ten minute mark in the incubation. The final points from 10 minutes to 60 minutes were terminated at 10 minute intervals. Points represented are the mean of duplicate samples.

Chart 5. PDD effect on rQT_3 uptake. Confluent cell monolayers (passage 3) in 35 mm dishes were covered with 1 ml of media containing 1 μM rQT_3 and further supplemented with 100 nM PDD (▲), 100 nM 4 α-PDD (△), 1 μM queuine (0), or unsupplemented control (●). Points represent the mean of duplicate samples, following the conditions outlined in Methods.

Chart 6. Lineweaver-Burk analysis for inhibition of rQT_3 uptake by 100 nM PDD. All data points are the mean of duplicate samples for one hour incubations at 37°C, following the conditions outlined in Methods. Symbols represent control (●) and 100 nM PDD (○) treated cells (passage 3) across a 100 fold concentration range of rQT_3 (10-1000 nM).

Chart 7. Combined PDD and quercetin effect on rQT_3 uptake. Confluent cell monolayers (passage 4) in 35 mm dishes were covered with 1 ml of media containing 100 nM rQT_3 and further supplemented with 100 nM PDD (▲), 0.1 mM quercetin (0), 100 nM PDD plus 0.1 mM quercetin (△), or unsupplemented controls (●). Points represent the mean of duplicate samples following conditions outlined in Methods.

Chart 8. Efflux of rQT_3 from preloaded cells. Confluent cell monolayers (passage 4) in 35 mm dishes were covered with 1 ml of media containing 1 μM rQT_3 for one hour at 37°C. The monolayers were rinsed quickly with media covered with 2 ml of unlabeled media, then incubated further at 37°C until terminated and rinsed as described in Methods. Following this the cell associated rQT_3 was extracted with ethanol. Part A represents efflux media supplemented with 10 μM dipyridamole (△), 100 nM PDD (▲), or unsupplemented controls (●). Part B represents identical circumstances, except that 100 nM PDD was present from the beginning of the 60 minute preloading step. Points represent the mean of duplicate samples following conditions outlined in Methods.

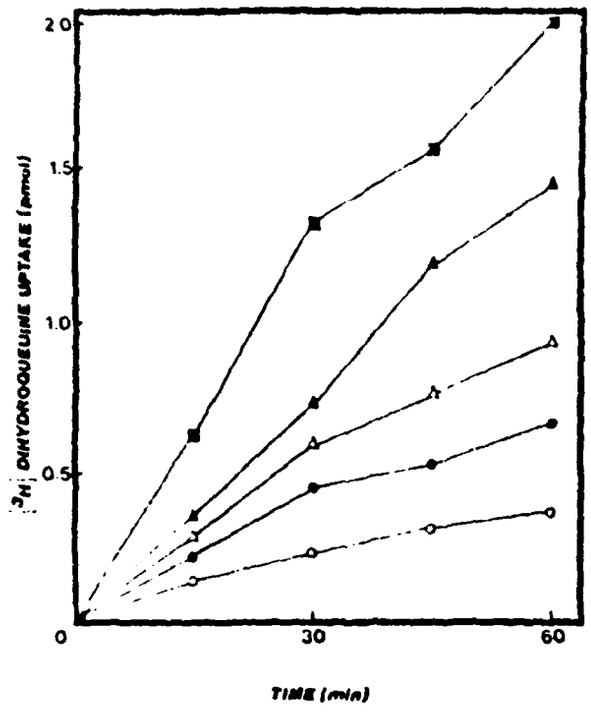
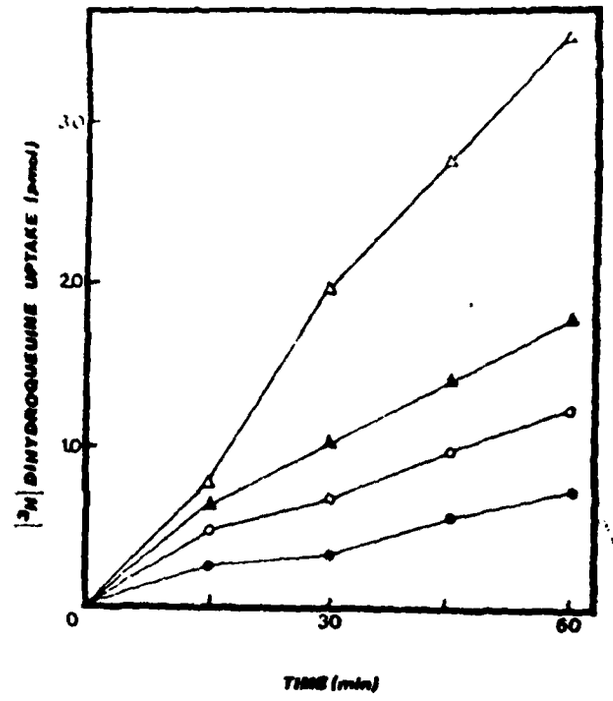


Chart 1



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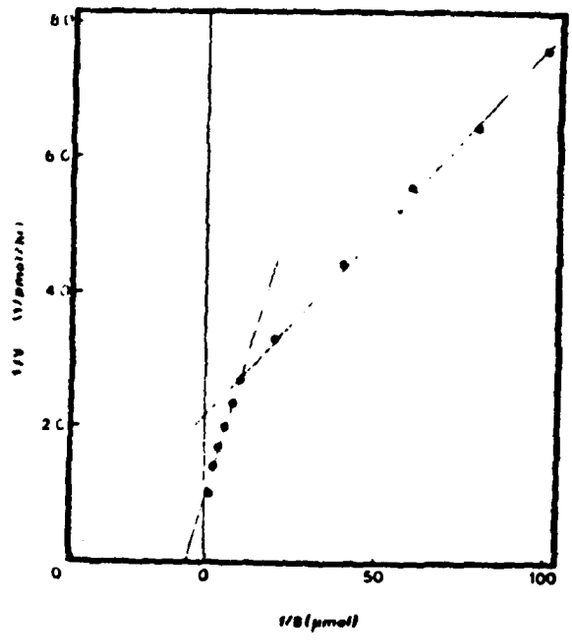

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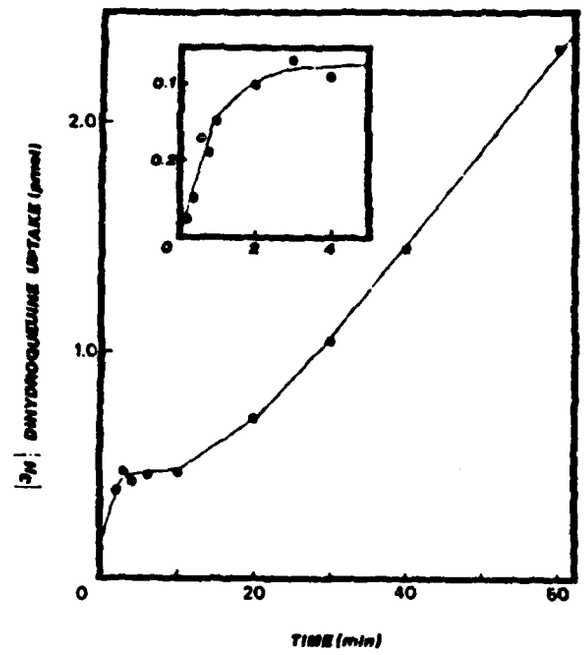
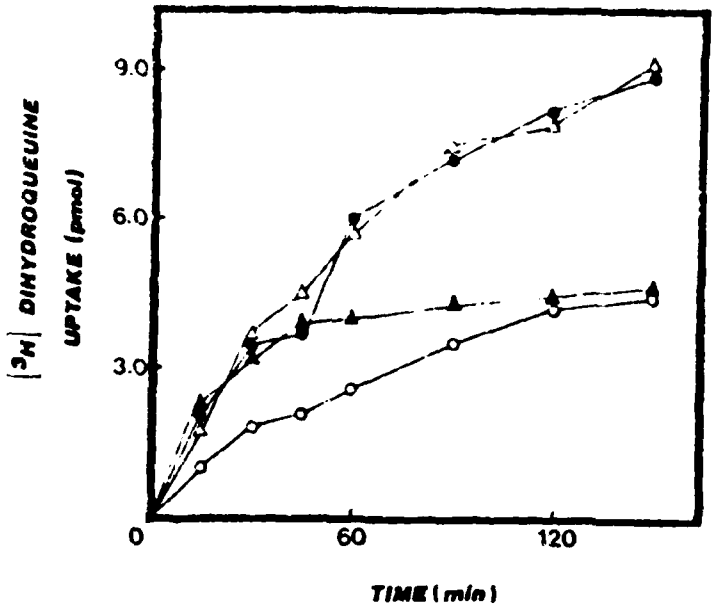


Chart 4



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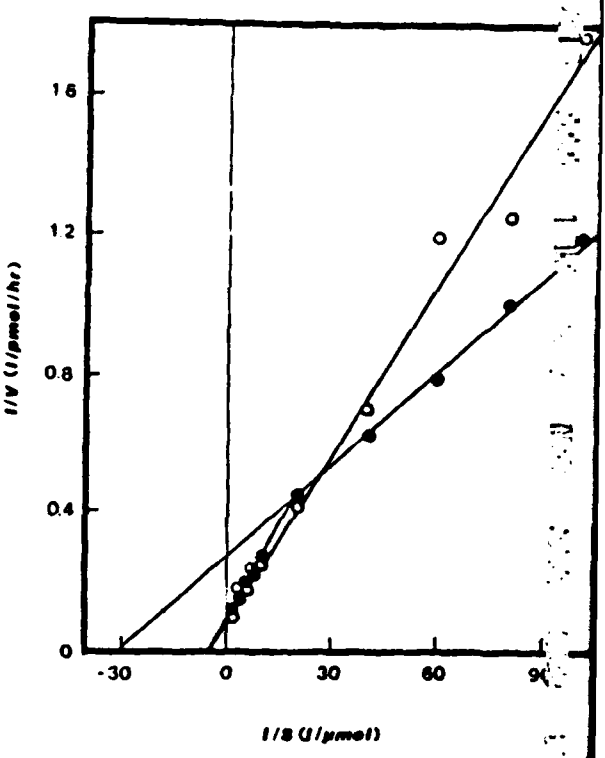

Chart 6

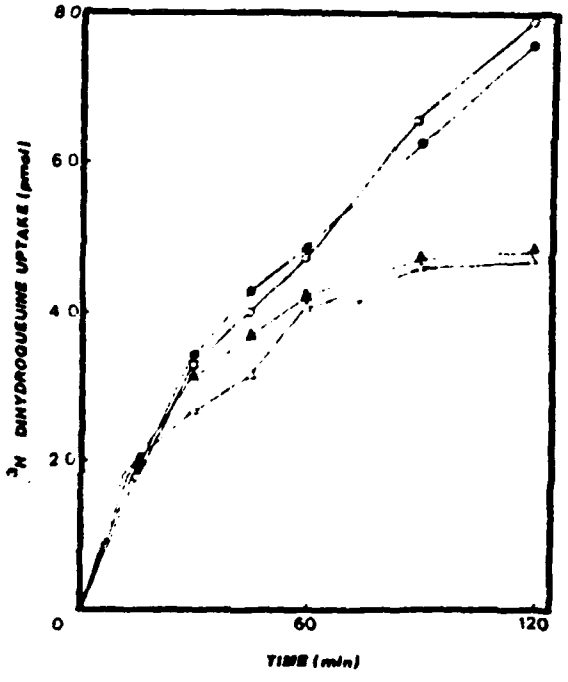

Chart 7

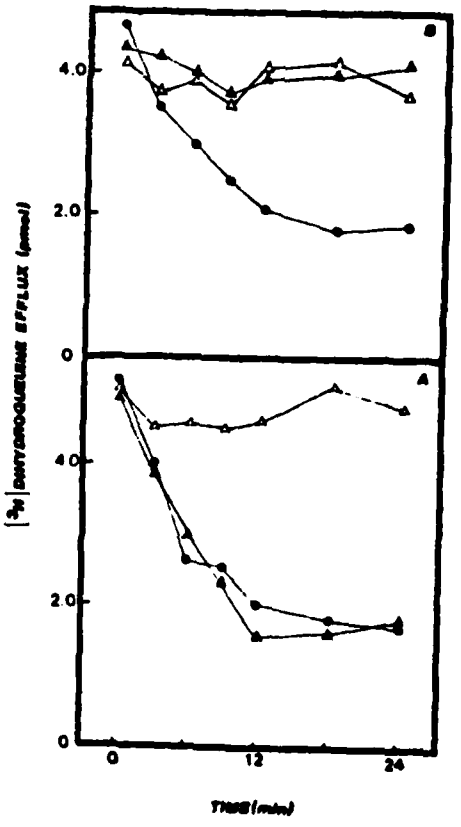

Chart 8

Proc. Soc. Exp. Biol. Med. (submitted)

Hematopoiesis and the Inosine Modification in Transfer RNA[1]

(Immunology)

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Abstract

Human promyelocytic leukemia (HL-60) cells were used to begin to evaluate the role in hematopoiesis of inosine biosynthesis in the tRNA anticodon wobble position; a reaction involving the enzymatic insertion of preformed hypoxanthine. Dimethylsulfoxide (DMSO) and hypoxanthine were found to induce the differentiation of HL-60 cells in a synergistic manner, and the induced differentiation was independent of changes in the purine catabolic enzymes adenosine deaminase and purine nucleoside phosphorylase. The short-term exposure of HL-60 cells to DMSO plus hypoxanthine resulted in enhanced leucine incorporation, and a model is presented showing how the inosine modification reaction in tRNA may be involved. A means by which hypoxanthine insertion into tRNA may modulate the synthesis of regulatory proteins (e.g. lymphokines and cell surface receptors) is also outlined.

Introduction

It had long been assumed that the nucleoside inosine found in the first position of the anticodon of specific tRNAs was generated in the macromolecules by the selective enzymatic deamination of adenosine (1). However, our recent demonstration of an enzymatic insertion of preformed hypoxanthine into tRNA (2) indicates that inosine biosynthesis occurs by a base exchange mechanism similar to that catalyzed by tRNA-guanine ribosyltransferase (EC 2.4.2.29) (3,4). The ramifications of this biosynthetic mechanism could be significant, since defects in the purine catabolic pathway responsible for generating hypoxanthine *in vivo* are associated with defects in cell-mediated immunity (5,6). Therefore, the absence of adenosine deaminase (EC 3.5.4.4) or purine nucleoside phosphorylase (EC 2.4.2.1) may interfere with a tRNA modification reaction of importance for immune function by blocking formation of the required substrate hypoxanthine.

Hypoxanthine also induces the differentiation of murine erythroleukemia cells *in vitro*, while the further catabolites xanthine and uric acid do not (7). The hypoxanthine-induced differentiation does not involve salvage into the cellular nucleotide pool (7), and in addition, no changes in purine salvage were observed during differentiation of these cells induced by dimethylsulfoxide (DMSO) (8). However, major changes in tRNA isoaccepting species were seen soon after inducing differentiation of cultured murine erythroleukemia cells with DMSO (9), and these included changes in some species ($tRNA^{Ala}$, $tRNA^{Leu}$, $tRNA^{Pro}$, $tRNA^{Ser}$ and $tRNA^{Thr}$) with the potential for being modified to contain inosine. Whether the macromolecular structural alterations actually involved the inosine modification was not established.

While a specific function in differentiation and/or cell-mediated immunity has not been demonstrated for inosine biosynthesis in tRNA, a role has been postulated (10). Inosine in the first position of the anticodon of a tRNA expands the codon recognition potential of that tRNA (11). Based on the wobble hypothesis for codon-anticodon pairing (11) and assuming inosine biosynthesis involves the exchange of hypoxanthine only for adenine (2), the codon reading capability should increase by 3-fold, i.e., inosine should be able to interact with uridine, cytidine, or adenosine in the third position of the mRNA codon while adenosine should only interact with uridine (11). Therefore, the inosine modification reaction was predicted to be required for mRNA codon translation involved in the synthesis of proteins and/or peptides of importance during hematopoiesis and in cell-mediated immunity (10).

A model depicting how inosine biosynthesis in tRNA might regulate protein synthesis is shown in Fig. 1. In the example illustrated, a leucine tRNA is modified to contain inosine, and that tRNA should be able to read the three leucine codons depicted in bold print (CUC,CUA, and CUU). The unmodified tRNA (with adenosine in the wobble position) should only read the last leucine codon (CUU), so the ribosome would stall (thereby blocking translation of the mRNA) if the anticodon modification were not carried out and leucine tRNAs capable of reading both of the other codons (CUC and CUA) were not present. Regulating protein synthesis by this means would be possible only because the genetic code is degenerate, so other essential mRNAs could use codons not read by inosine-containing tRNAs. For example, different mRNAs in the same cell (or cell compartment) depicted in Fig. 1 could make use of the leucine codons CUG, UUA, and UUG, and as a result, their ability to function in protein synthesis would not be restricted.

The model illustrated in Fig. 1 offers the basis for our studies of the potential role of inosine biosynthesis in tRNA in regulating (or modulating) hematopoiesis and cellular immunity. The intracellular availability of hypoxanthine is predicted to be a controlling factor as to whether inosine biosynthesis occurs and, therefore, whether specific mRNAs are translated.

Materials and Methods

Cell culture. Human promyelocytic leukemia (HL-60) cells, obtained from Dr. Robert Gallo at the National Cancer Institute, were grown in suspension culture in RPMI-1640 medium supplemented with 15% fetal bovine serum. Growth curves were established after HL-60 cells were plated at 6×10^4 cells/ml in 10 ml of medium per 25 cm^2 culture flask. Control cultures and cultures treated with 100 mM DMSO and/or 1 mM hypoxanthine were maintained in duplicate, and cells were enumerated with a Coulter Counter. Based on the hypoxanthine content of the lot of serum utilized, the final concentration of hypoxanthine in the control and DMSO containing medium was 12 μM. Cytospin preparations were used to assess morphological differentiation of the HL-60 cells treated as described above. The cells were stained with Wright-Giemsa, and differential cell counting was performed on a minimum of 200 cells to determine the percent of total cells exhibiting mature morphology (13).

Enzyme assays. HL-60 cells in 1 l spinner flasks were harvested by low speed centrifugation, washed, and homogenized in reticulocyte standard buffer (RSB). The extract was then centrifuged at 30,000 x g for 20 min at 4° C, and the supernatant was used to assay adenosine deaminase and purine nucleoside phosphorylase. In both cases, a spectrophotometric, coupled enzyme system was

used to monitor uric acid formation (14,15). For adenosine deaminase, the standard reaction mixture contained 100 mM phosphate buffer (pH 7.2), 1.5 mM adenosine, 20 units of purine nucleoside phosphorylase, 20 units of xanthine oxidase, and cell extract in a total volume of 1 ml. For purine nucleoside phosphorylase, the same reaction mixture was used except 0.4 mM inosine replaced adenosine as the substrate and the commercial purine nucleoside phosphorylase was omitted. Control reactions contained everything except substrate or cell extract. The reaction mixtures were incubated at 37° C, and uric acid formation was monitored as an increase in absorbance at 293 nm using a Beckman DU-8 recording spectrophotometer. An extinction coefficient of 12.1/μmole/cm^3 for uric acid was used to calculate product formation, with units of enzyme activity in nmole product/min.

Leucine incorporation. HL-60 cells were harvested by low-speed centrifugation, washed twice with a Hank's balanced salt solution, and resuspended at 5 x 10^5 cells/ml in Hank's alone or Hank's supplemented with 210 mM DMSO, 1 mM hypoxanthine, or 210 mM DMSO plus 1 mM hypoxanthine. After preincubating at 37°C for 10 min, [^{14}C]leucine (50 mCi/mmole) was added at 0.5 μCi/ml. At various time intervals up to 60 min, the amount of acid-insoluble radioactive leucine incorporated was determined by precipitation of 0.5 ml aliquots with 2.5 ml of 10% trichloroacetic acid (TCA) (16,17). After 30 min on ice, the precipitates were collected on glass fiber filters, washed with 40-50 ml of ice-cold 5% TCA, dried, and counted by liquid scintillation.

Materials. Fetal bovine serum (Lot No. 100430) was obtained from Sterile Systems, Logan, UT, while the cell culture medium RPMI-1640 and the Hank's balanced salt solution were from GIBCO, Grand Island, NY. Purine nucleoside phosphorylase and xanthine oxidase were purchased from Boehringer Mannhein,

Indianapolis, IN, while the [^{14}C]leucine was purchased from Amersham, Arlington Heights, IL.

Results

Growth curves for HL-60 cells treated with DMSO and hypoxanthine alone and in combination are shown in Fig. 2. Treatment with 100 mM DMSO alone diminished the growth of the HL-60 cells only slightly compared to the untreated controls, while 1 mM hypoxanthine was somewhat more effective. However, the combination of 100 mM DMSO plus 1 mM hypoxanthine resulted in an almost complete cessation of growth.

Although 1 mM hypoxanthine inhibited the growth of HL-60 cells, it was not effective at inducing differentiation (Table 1). Likewise, 100 mM DMSO alone caused no change in the proportion of cells exhibiting a more mature morphology when compared to the untreated controls. However, the combination of DMSO and hypoxanthine yielded a significant population of cells more mature than promyelocytes (Table 1).

Adenosine deaminase and purine nucleoside phosphorylase were assayed in extracts of untreated HL-60 cells as well as in HL-60 cells induced to differentiate with DMSO plus hypoxanthine. The specific activity of purine nucleoside phosphorylase was almost 3-fold higher than adenosine deaminase in the HL-60 cells, but no differences were seen for the treated versus untreated cultures (Table 2). Results similar to those for the 24 hr exposure to DMSO plus hypoxanthine were obtained at 72 hr (data not presented).

Radiolabelled leucine incorporation into TCA precipitable material was monitored with HL-60 cells treated short-term with DMSO plus hypoxanthine

(Fig. 3). Compared to untreated control cells, these cells incorporated appreciably more leucine. The combination of DMSO and hypoxanthine also yielded significantly more leucine incorporation than did DMSO alone or DMSO plus adenine. In separate experiments, 1 mM hypoxanthine alone was found to have no effect (data not presented).

Discussion

It has been known for sometime that, individually, DMSO and hypoxanthine will induce the differentiation of HL-60 cells (18). However, the concentrations required are higher than those utilized in much of this investigation. It was surmised that the mechanism of action for DMSO-induced differentiation might involve enhanced hypoxanthine transport into the cell or cell compartment where inosine modification in tRNA occurs (Fig. 1). Therefore, suboptimal concentrations of DMSO and hypoxanthine were evaluated in combination, and a synergistic induction of HL-60 differentiation was observed (Fig. 2 and Table 1). While these results do not prove the mode of action of either agent, they are consistent with the postulated model.

Changes in purine catabolism are common during hematopoiesis, but the function of such changes remains obscure. Significant 2 to 3-fold increases in purine nucleoside phosphorylase activity (without a change in adenosine deaminase) were reported during the phorbol ester-induced differentiation of chronic lymphocytic leukemia cells (19), while nearly a 10-fold increase in adenosine deaminase activity was reported when peripheral blood monocytes differentiated into macrophages in vitro (20). In the latter case, inhibiting the elevation of adenosine deaminase activity blocked the cellular

differentiation, thereby indicating a fundamental role for the enzyme. No such differences in the purine catabolic enzymes were observed in the present study when HL-60 cells were induced to differentiate with DMSO plus hypoxanthine (Table 2). However, if the increases in purine catabolism are needed to generate the substrate for inosine biosynthesis in tRNA (Fig. 1), such changes should not be required when the substrate hypoxanthine is supplied exogenously.

Enhanced protein synthesis (based on radiolabelled leucine incorporation) was reported during the differentiation of HL-60 cells in vitro (17). In addition, the leucine radiolabelling of specific membrane-associated proteins was increased significantly during HL-60 differentiation into macrophage-like cells induced by phorbol esters (21). These data are consistent with the model presented in Fig. 1, and further support was obtained in this investigation (Fig. 3). The short-term (10 min) exposure of HL-60 cells to DMSO plus hypoxanthine prior to adding radiolabelled leucine resulted in a significant increase in leucine incorporation into TCA-precipitable material. Neither DMSO alone nor DMSO plus adenine (a purine which should not effect an increase in tRNA inosine biosynthesis) caused similar increases. Considering our previous demonstration that immature leukocytes have very little inosine in their tRNA (10), the results in Fig. 3 might best be explained by hypoxanthine insertion into leucine tRNAs which should enhance radiolabelled leucine incorporation into polypeptides as depicted in Fig. 1.

As a means for regulating gene expression, the model in Fig. 1 is somewhat analogous to the attenuation mode described for various bacterial operons (22). With that system, the level of aminoacylation of a particular tRNA determines whether ribosomes stall or translate leader sequences preceding structural genes in mRNA. The model presented here relies instead on a post-transcriptional

structural modification in the tRNA anticodon. This modification is predicted to allow stalled ribosomes to proceed, thereby releasing a block in protein synthesis. Since the hypoxanthine insertion reaction expands the wobble capability of the tRNA, this type of control might be described as wobble attenuation of translation. Again, such a form of regulation would be possible only because the genetic code is degenerate, so other essential mRNAs could use different codons which are read by tRNAs not subject to similar controls.

An interesting possibility for how wobble attenuation of translation might be involved specifically in the control of hematopoiesis or immune function deals with the translocation of regulatory proteins through or into the cell membrane. In many such cases, the initial translation product has a signal peptide sequence at the amino terminus which allows the translocation of the protein (23). The signal peptide is then removed during or after the translocation process. Examination of the leucine codons in signal sequences for two T-cell proteins [interleukin-2 (T-cell growth factor) and a cell surface receptor polypeptide] demonstrates a preponderance of codons (CUU, CUC, CUA) with the potential to be read by an inosine-containing leucine tRNA (Table 3). Four of 5 leucine codons in the interleukin-2 signal sequence could be translated by this tRNA, while only 5 of 18 leucine codons in interleukin-2 itself could be (24), i.e., other leucine tRNAs would be required to read 13 of the latter codons (CUG, UUA, UUG). The situation for the receptor polypeptide is even more polarized where 6 of 6 leucine codons in the signal sequence could be read by the inosine-containing tRNA while only 6 of 21 in the remainder of the mRNA could be (25). Therefore, the synthesis of such proteins might be regulated, at least in part, by wobble attenuation of translation in the signal sequences as depicted in Fig. 1. The data reported here for radiolabelled

leucine incorporation (Fig. 3) and that previously published (17,21) appear to be compatible with such a model. The implications of this mechanism with regard to hematopoiesis and immune function could be far reaching, since the tRNA modification reaction may be subject to modulation by extrinsic agents.

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Table 1. *Differential* counts of HL-60 cells after incubation with dimethylsulfoxide (DMSO) and/or hypoxanthine (Hx).[1]

Inducer	Percent of Total Cells			
	Promyelocytes	Myelocytes	Metamyelocytes	Neutrophils
None	89.4	7.8	2.8	0
DMSO	89.6	6.3	4.1	0
Hx	97.1	2.5	0.4	0
DMSO/Hx	46.2	32.4	19.5	1.9

[1] The cells were treated for 9 days with 100 mM DMSO, 1 mM Hx, or 100 mM DMSO plus 1 mM Hx. See Fig. 2 and the Materials and Methods section for additional details.

Table 2. Adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) activity in HL-60 cells after incubation with dimethylsulfoxide (DMSO) and hypoxanthine (Hx).[1]

Inducer	Specific Activity[2]	
	ADA	PNP
None	55.4 ± 3.5	139 ± 9
DMSO/Hx	51.1 ± 4.3	143 ± 26

[1] The cells were treated for 24 hours with 210 mM DMSO plus 1 mM Hx, after which ADA and PNP were assayed in crude cell homogenates as described in Materials and Methods.

[2] The results indicate the mean (± S.D.) of duplicate determinations from two independent experiments and are expressed as nmol/min/mg protein.

Table 3. Leucine codons in signal sequences for T-cell proteins.[1]

		Potential Anticodon Recognition[3]						
Position[2]	Codon	CAA	UAA	CAG	(AAG)	UAG	(GAG)	IAG
Interleukin-2:								
S6	CUC						(X)	X
S7	CUG			X		X		
S12	CUA					X		X
S14	CUU				(X)		(X)	X
S16	CUU				(X)		(X)	X
Receptor Polypeptide:								
S5	CUU				(X)		(X)	X
S6	CUC						(X)	X
S7	CUC						(X)	X
S11	CUA					X		X
S13	CUU				(X)		(X)	X
S18	CUC						(X)	X

1 Based on nucleotide sequences published by Taniguchi et al. (24) for human interleukin-2 and Hedrick et al. (25) for a mouse T-cell receptor polypeptide.

2 The AUG initiator codon is designated as position number S1. The signal sequences for interleukin-2 and the receptor polypeptide code for 20 and 19 amino acids respectively.

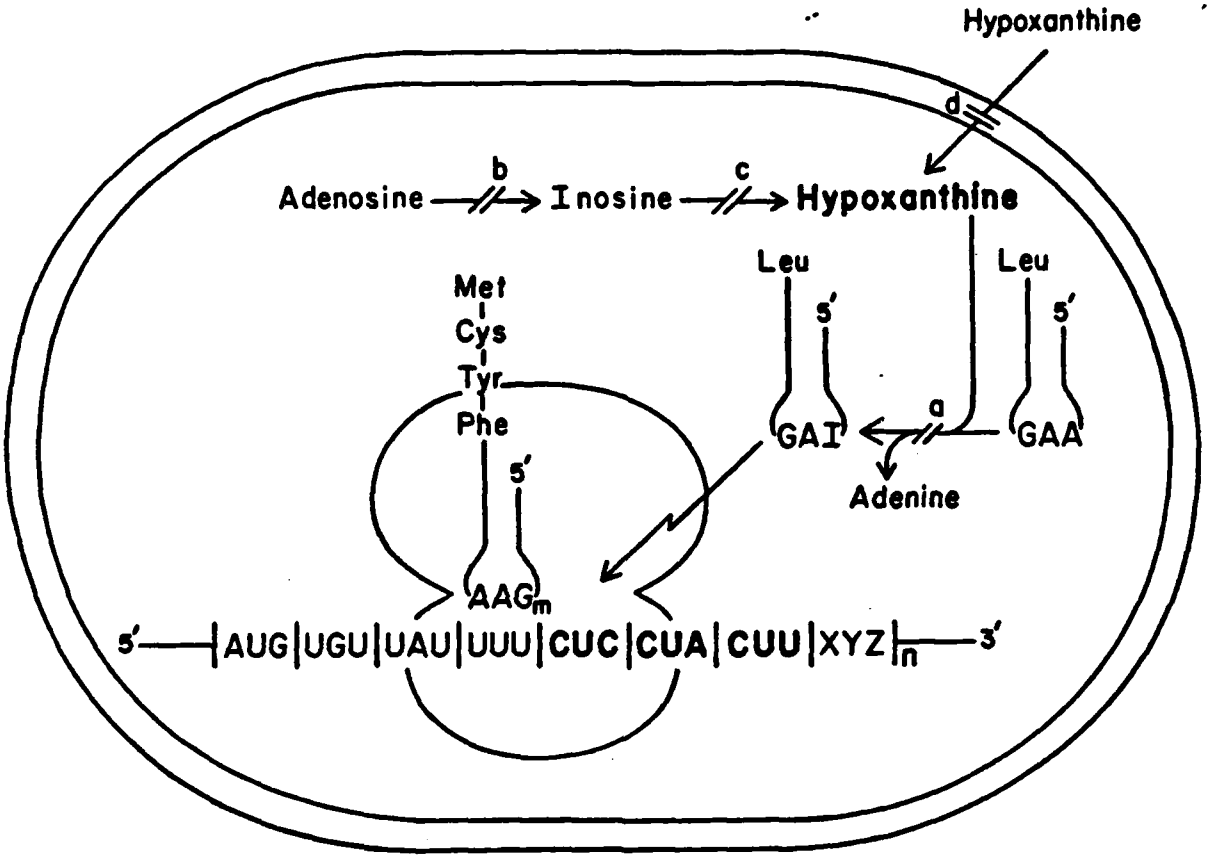
3 Based on the wobble hypothesis of Crick (11). All possible leucine tRNA anticodon base sequences are included. The anticodons in parentheses have not been reported in eukaryotic tRNAs (12). The anticodons (and codons) are written using the standard 5'--->3' orientation.

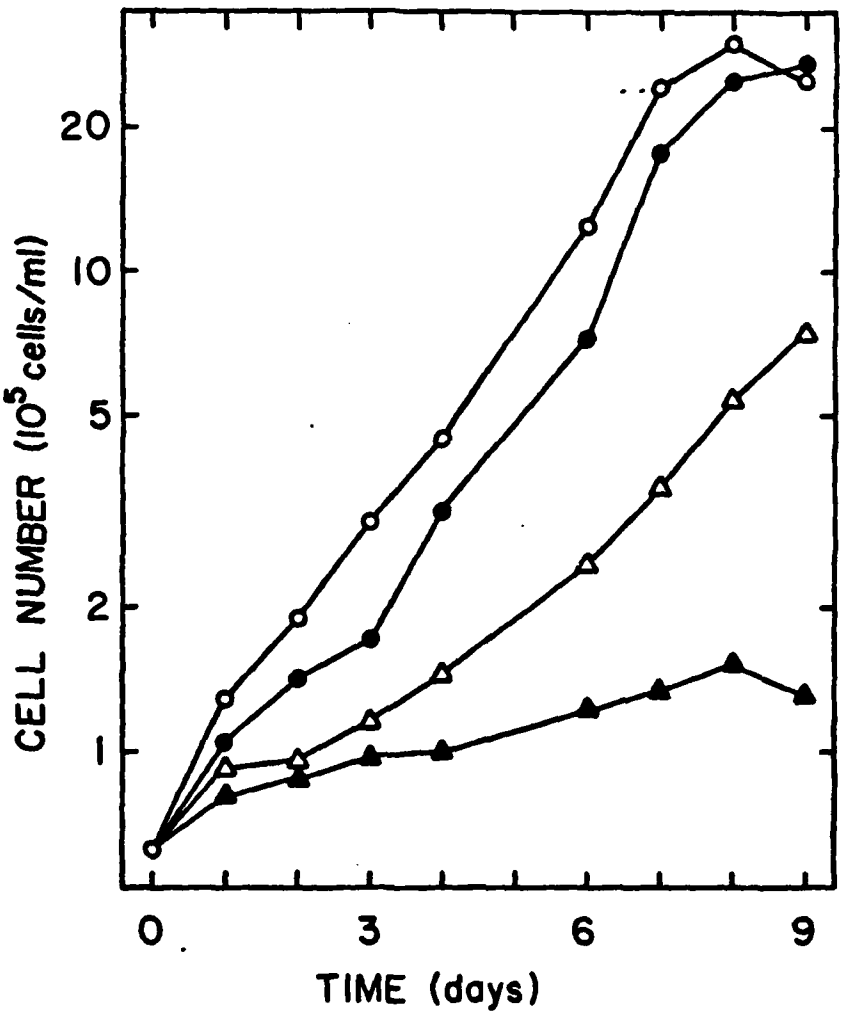
Figure Legends

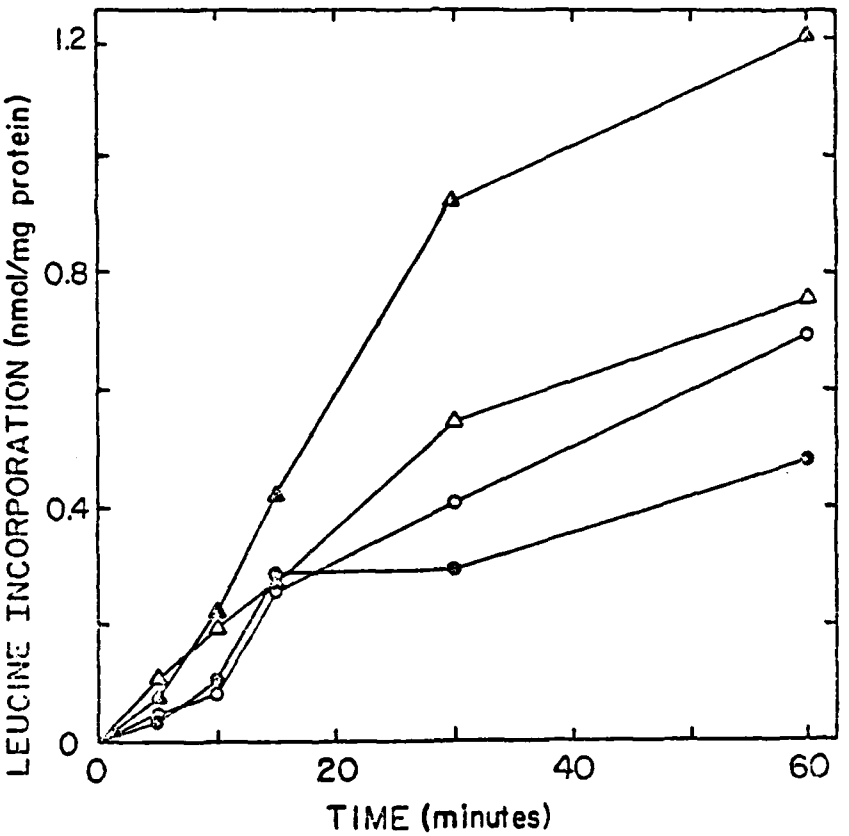
Fig. 1. Postulated model for how the hypoxanthine insertion reaction in specific tRNA anticodons may regulate protein synthesis. Transfer RNAs with the potential for having hypoxanthine inserted into the first position of the anticodon include those for alanine, arginine, isoleucine, leucine, proline, serine, threonine, valine, and perhaps glycine (2,12). Any of these tRNA species with adenine in the primary transcript wobble position could be involved in regulating translation as shown. However, in the example illustrated, a leucine tRNA is modified by tRNA-hypoxanthine ribosyltransferase (a) which, according to the wobble hypothesis (11), should allow the resulting inosine-containing tRNAs to read the three leucine codons depicted in bold print (CUC, CUA, and CUU). The unmodified tRNAs should only read the last leucine codon (CUU), so the ribosome would stall if the anticodon modification were not carried out. The substrate for inosine biosynthesis in tRNA, hypoxanthine, could be generated endogenously from adenosine and inosine by the enzymes adenosine deaminase (b) and purine nucleoside phosphorylase (c), or it could be supplied exogenously and transported (d) into the cell or cell compartment.

Fig. 2. HL-60 growth curves. Cells plated at an initial density of 6×10^4 cells/ml were monitored for 9 days as described in Materials and Methods. The curves depict untreated control cells (O) as well as cells treated with 100 mM DMSO (●), 1 mM hypoxanthine (Δ), and 100 mM DMSO plus 1 mM hypoxanthine (▲).

Fig. 3. Enhanced leucine incorporation by HL-60 cells treated with DMSO plus hypoxanthine. Cells (5×10^5/ml) preincubated for 10 min in a Hank's balanced salt solution alone (O) or Hank's plus 210 mM DMSO (●), 210 mM DMSO and 1 mM adenine (Δ) or 210 mM DMSO and 1 mM hypoxanthine (▲) were radiolabelled with [^{14}C]leucine starting at time zero. At the time intervals indicated, samples were removed, and the amount of covalently incorporated leucine was determined by acid precipitation. See Materials and Methods for additional details.


Hypoxanthine
d
Adenosine
b
Inosine
c
Hypoxanthine
Leu
Leu
Met
Cys
Tyr
Phe
5'
5'
5'
5'
a
GAI
GAA
Adenine
AAG$_m$
5'
AUG|UGU|UAU|UUU|CUC|CUA|CUU|XYZ$|_n$
3'


CELL NUMBER (10^5 cells/ml)
20
10
5
2
1
0
3
6
9
TIME (days)


LEUCINE INCORPORATION (nmol/mg protein)
1.2
0.8
0.4
0
0
20
40
60
TIME (minutes)

END

FILMED

4-85

DTIC